APPLICATION OF SELECTIVE METHODS IN THE SEARCH FOR NEW BIOACTIVE NATURAL PRODUCTS FROM FUNGI

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Abstract

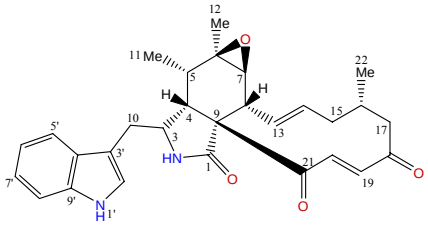
Fungi are prolific sources of structurally novel and biologically active compounds. Over the past decades terrestrial fungi have been a major source of bioactive metabolites. But often these have been obtained from easily accessible and culturable organisms while the apparently rare and non-culturable groups remain an untapped resource. With the need for new bioactive compounds increasing, the yield of potentially useful new bioactive natural products from microbial sources is declining. Part of this problem stems from the repeated discovery of known compounds due to the heavy reliance on soil actinomycetes and common soil fungi as the source of choice.

A study was made using a range of phenol concentrations as a selective method for the isolation of ascomycetes from New Zealand soils. A total of 407 isolates were obtained. Culture extracts of a number of these isolates showed significant activity against the P388 murine leukemia cell line, *Bacillus subtilis* and *Candida albicans*.

Chemical characterization of metabolites in active extracts was greatly aided by employing dereplication techniques using a combination of HPLC-UV/R_t database, MS analysis, the CapNMR technique and the AntiMarin Database. A significant advantage was gained by the use of the

CapNMR technique that enabled a full NMR characterization on very small quantities (<20 µg) of the pure compound.

Of these active extracts, one from *Pseudeurotiom zonatum* exhibited P388 activity with an IC_{50} value of 1,035 ng/mL and two new cytochalasins; F7301-1 (**A**) and F7301-2 (**B**) were subsequently isolated.



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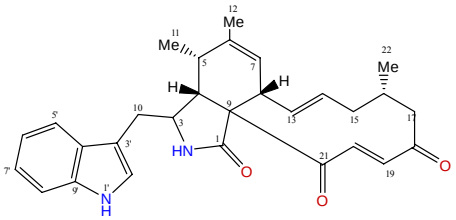

B

This isolation strategy for the characterisation and exploitation of the less readily culturable organisms greatly influences the potential for the discovery of novel biotypes and new bioactive compounds.

As well as exploring biodiversity with the isolation of less culturable fungi an attempt was made to maximize metabolite production and induce the production of novel metabolites from active selected fungi. This strategy was based on the OSMAC (one strain many compounds) and hormetic (low-dose stimulation and high-dose inhibition) concept. In the OSMAC studies the secondary metabolite production was greatly affected by subinhibitory concentrations of elicitors for three out of the seven chosen fungal isolates. These were *Chaetomium globosum*, *Xylaria* sp. and

Bombardia sp. *Pseudeurotium zonatum* only showed an enhancement in metabolite productions but not modulation. The remaining three isolates, *Chaetomium trigonosporum*, *Penicillium* sp and isolate 901_7@20.14, were not influenced by manipulation or produced no metabolites at all under the range of conditions and elicitors used.

Abbreviations

%	percent
°C	degrees Celsius
1D	one dimensional
2D	two dimensional
δ	chemical shift in ppm (in NMR)
λ_{max}	maximum wavelength in nm
µg	microgram
µL	microlitre
µm	micrometer
µM	micromolar
ACN	acetonitrile
amu	Atomic Mass Unit
Au-Pt	Gold Palladium
B.C.	Before Christ
br	broad (in NMR)
C18	octadecyl phase (chromatography column packing)
CapNMR	capillary-probe NMR
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CLY	cycloheximide
COSY	correlation spectroscopy (in NMR)

d	doublet (in NMR)
DAD	diode array detector
DCM	dichloromethane
DEA	diethylamine
DEPT	distortionless enhancement by polarization transfer (in NMR)
DMDCS	dimethyldichlorosilane
DMSO-d6	deuterated dimethyl sulfoxide
e.g.	example given
EI	electron ionization
ELSD	evaporative light scattering detector
ESI	electrospray ionization
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
F-actin	filamentous actin
g	gram
h	hour
HDACI II	Histone Deacetylase Inhibitor II
HDACI IV	Histone Deacetylase Inhibitor IV
HMBC	heteronuclear multiple bond correlation spectroscopy (in NMR)
H_2O	water
HPLC	high performance liquid chromatography
HRESIMS	high resolution electrospray ionization mass spectrometry

HRLCMS	high resolution liquid chromatography mass spectrometry
HSQC	heteronuclear single quantum coherence (in NMR)
Hz	hertz
i.e.	*id est* (that is)
IC_{50}	concentration of sample required to inhibit the growth of P388 cellgrowth by 50%
J	coupling constant in Hz (in NMR)
JAS	jasplakinolide
kV	kilovolt
LB	latrunculin B
LCMS	liquid chromatography mass spectrometry
log	logarithmic
m	multiplet (in NMR)
mA	milliampere
MeOH	methanol
mg	milligram
mL	millilitre
mm	millimeter
mM	milliMol
mV	millivolt
MeOH	methanol
MHz	megahertz
min	minute

MPA	mycophenolic acid
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYP	Malt Yeast Peptone
MYPA	Malt Yeast Peptone Agar
MYPB	Malt Yeast Peptone Broth
m/z	mass-to-charge ratio (in MS)
n	number of repeating subunit
ng	nanogram
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect (in NMR)
No.	number
NYS	nystatin
OD	Optical density
OSMAC	one strain-many compounds
pA	picoampere
psi	pound per square inch
P388	cells murine leukaemia cells
PDA	photodiode array
PHA	phalloidin
PHB	poly-3-hydroxybutyrate
ppm	parts per million

PTFE	polytetrafluoroethylene
q	quartet (in NMR)
R_t	retention time
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640 medium
s	singlet (in NMR)
SDA	Sabouraud Dextrose Agar
SDA^+	Sabouraud Dextrose Agar plus antibiotic solution
¼SDA-CS	¼ strength sabouraud dextrose agar-chlortetracycline/streptomycin medium
SDB	Sabouraud Dextrose Broth
SEM	scanning electron microscope
Sol^n	solution
sp.	species (singular)
t	triplet (in NMR)
TCZ	tricyclazole
Temp	temperatures
TTC	tetracycline
TFA	trifluoroacetic acid
UV	Ultra-Violet
V	volts

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CHAPTER 1

INTRODUCTION

1.1 NATURAL PRODUCTS OVERVIEW

Natural products are organic compounds that are formed by living organisms. These naturally occurring compounds can be divided into three broad categories:

a) Primary metabolites. These compounds play a role

in the metabolism and reproduction of the cell and include amino acids, nucleic acids and sugars.

b) Structural components of the cell. For example, cellulose in plant cell walls (Hanson, 2003)

c) Secondary metabolites. Unlike the first two categories which have roles essential for life, secondary metabolites are not directly essential for the normal growth, development or reproducibility of the organism. The actual role of secondary metabolites in cellular systems has been greatly debated in recent years. The general consensus is that secondary metabolites serve roles related to survival functions in organisms that produce them (Demain and Fang, 2000), and that the production of secondary metabolites is affected by external stimuli such as environmental stress, competition and infection (Strohl, 2000).

Secondary metabolites are derived from biosynthetic pathways which branch off from the primary metabolic pathways (Benita, 1994). With the exception of high molecular mass compounds such as maitotoxin and palylatoxin, most of these natural occurring compounds have molecular masses less than 1,500 amu (Bentley, 1999; Cannell, 1998).

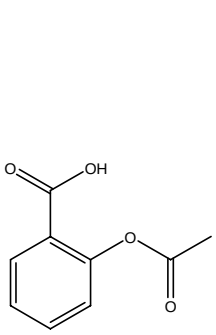
1.2 NATURAL PRODUCTS AS A SOURCE OF MEDICINES

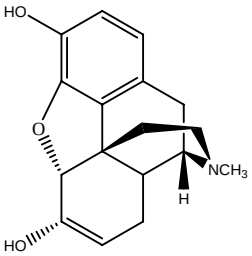
1.2.1 History of natural products discovery

Natural products from plants and fungi have been used by man for thousands of years as traditional medicines and as natural poisons and are recognised as an important source of therapeutically effective drugs (Larsen *et al*. 2005). In the past, the plant kingdom has served as the main source of natural products. The first records were written in on clay tablets in cuneiform from Mesopotamia and date from about 2600 B.C. The Ebers papyrus, which is the Egyptian pharmaceutical record, date from about 1500 B.C. and described around 700 drugs (mostly from plants) including formulae for gargles, snuffs, infusion, pills and ointments. Since then the use of natural products has been widely recorded across all continents (Cragg and Newman, 2005). Today natural products still serve a role directly as traditional medicines and the largest users are the Chinese. Over 5,000 natural products have been recorded in their pharmacopeia (Bensky and Gamble, 1993).

These traditional medicines, which again derive mostly from plants, formed the basis of most of the early medicines (Butler, 2004).

The most universally used and the world's best known drug is aspirin (**Figure 1.1**), which was originally derived from willow trees and other salicylate-rich plants. The earliest reference of the use of willow bark extract as an antipyretic to reduce fever and act as an anti-inflammatory medication comes from the Ebers Papyrus, an Egyptian medical text from 1543 BC (Diarmuid, 2005). Other examples of natural products with a long history of use include morphine (**Figure 1.2**), an opioid analgesic which was isolated from the opium poppy; *Papaver somniferum*. Although known for thousands of years it has only recently been understood how the opioid analgesic effect of morphine operates at the molecular level (Aldrich, 1996). Another remarkable medicine of note is the anti-malaria drug quinine (**Figure 1.3**) derived from the cinchona tree, *Cinchona officinalis* and other *Cinchona* species native to South Africa (Casteel, 1997).





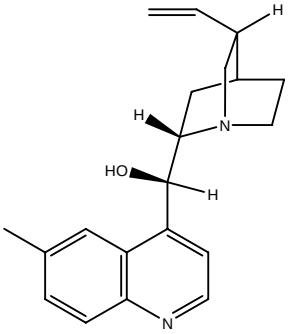


Figure 1.1: Aspirin **Figure 1.2:** Morphine **Figure 1.3:** Quinine

Natural products research and fungal metabolite studies were revolutionized by the discovery of penicillin in 1928 by Alexander Fleming from *Penicillium chrysogenum.* This "wonder drug" has literally saved millions of

lives and sparked an era of fungal-derived medicines (Tulp and Bohlin, 2004). Penicillin G (**Figure 1.4**), the original metabolite isolated, is still a front-line antibiotic for some common bacterial infections, although its effectiveness is now limited by the development of penicillin-resistant gram-negative bacteria.

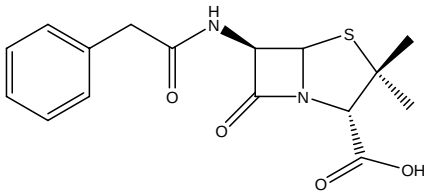
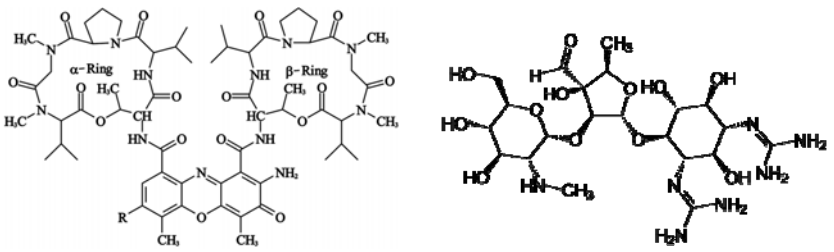


Figure 1.4: Penicillin G

The phenomenal success of penicillin led to the intensive search for other antibiotic-producing micro-organisms. The search was not limited to fungi, but also included bacteria and actinomycetes. For example, tyrothricin, a mixture of two antibiotics gramicidin and tyrocidin, was isolated from *Bacillus brevis* in 1939. Actinomycin was the first antibiotic isolated from actinomycetes in 1941, followed by streptomycin (**Figure 1.5**) which was isolated from *Streptomyces griseus* in 1942 (Benita, 1994). The concept of fermentation products as a productive source of new antibiotics was validated after the discovery of streptomycin in 1943 (Kirst, 1996).

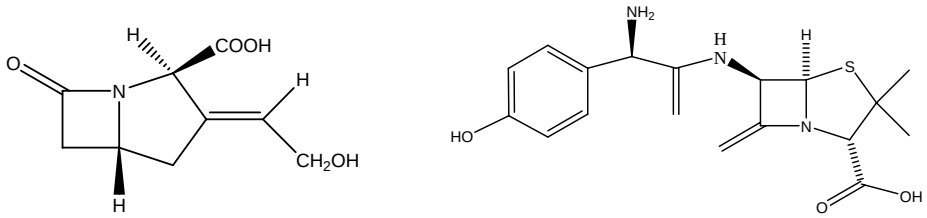


actinomycin streptomycin

Figure 1.5: Actionomycin and streptomycin

The clinical uses of microbial natural products are not limited to antibiotics, and as such antiviral and antifungal agents have also been developed (Strohl, 2000). Recent, newer developments of natural product-based drugs include anti-diabetic, anticancer, immunosuppressive, and anti-malaria drugs (Harvey, 2000).

Most of the natural products that enter pharmaceutical markets today are derived from terrestrial micro-organisms. A classic example would be "Augmentin" which is a combination of clavulanic acid and amoxicillin **(Figure 1.6).** These natural products are derived from *Streptomyces clavuligerus* and the *Penicillium nucleus* respectively.



clavulanic acid amoxicillin

Figure 1.6: Clavulanic acid and amoxicillin

Mevastatin **(Figure 1.7)** and lovastatin **(Figure 1.8)**, which were originally isolated from *Penicillium citrinum* and *Aspergillus terreus* respectively, were the lead compounds for many cholesterol-lowing agents that have combined annual sales of more than US$ 16 billion dollars in 1999 (Alberts *et al*. 1980; Butler, 2004; and Malik *et al*. 2006).

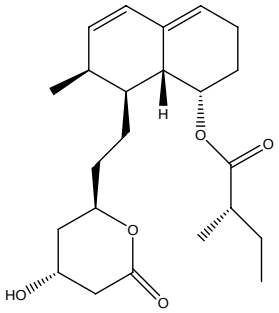


Figure 1.7: Mevastatin

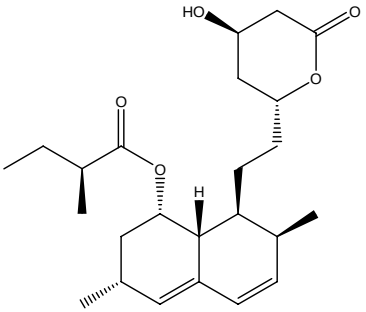


Figure 1.8: Lovastatin

1.2.2 Fungal secondary metabolites

Fungi are prolific producers of secondary metabolites. In 1971 Turner determined that there were almost 1,000 fungal products but this had more than doubled by the next decade (Turner and Aldridge, 1983). To date more than 6,000 different metabolites have been reported from fungi (Zain *et al*. 2009). Some of the fungal metabolites that have been known for a long time are finding new and unexpected uses in medicine today. A recent example is mycophenolic acid, (**Figure 1.9**) first isolated from the fungus *Penicillium brevicompactum* by Gorsio in 1896, for which the structure was not reported until 1952. Mycophenolic acid was developed as an immunosuppressive agent in 1995. In addition a synthetic pro-drug, mofetil (**Figure 1.10**) which is based on mycophenolic acid, as it liberates mycophenolic acid under physiological conditions, is now commercially available (Pearce, 1995).

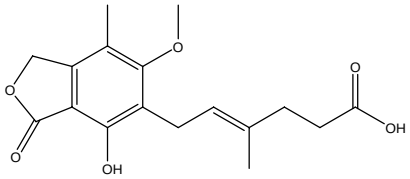


Figure 1.9: Mycophenolic acid

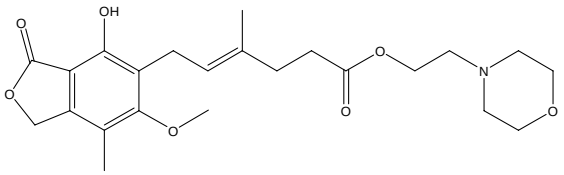


Figure 1.10: Mofetil

While the need for new bioactive compounds is increasing, the yield of potentially useful new bioactive natural products from microbial sources is declining (Perric-Concha and Long, 2003). Part of this problem stems from the repeated discovery of known compounds due to the heavy reliance on soil actinomycetes and common soil fungi as the source of bioactive metabolites.

In terms of biodiversity, fungi are the second largest group of organisms in the world after insects. Hawksworth (1991) estimated the actual number of fungal species could be up to 1.5 million. This is over five times the predicted number of plant species and 50 times the estimated number of bacterial species. However, less than 5% of the fungal species have so far been described and of this 5% only a fraction have been examined for natural product production. Among the fungi, the Ascomycetes represent the largest group of fungi, but they remain relatively unexplored (Kendrick, 1992).

Soil has been one of the most highly investigated sources of micro-organisms for the discovery of novel compounds because it carries a higher population of micro-organisms than any other habitat (Whitman *et al.* 1998). New Zealand has many diverse soil ecotypes ranging from hot muddy soils

in geothermally active regions to icy cold soils of mountains and glaciers. The soils of the temperate rain-forests also offer much potential in terms of microbial diversity.

1.3 ASCOMYCOTA

The phylum Ascomycota is the largest phylum of fungi with over 30,000 species (Hawksworth and Kalin-Arroyo, 1995). Both the Ascomycota and Basidiomycota share a number of important features such as a chitinous cell wall, formation of septate hyphae, hyphae fused with one another to exchange nuclei, and the presence of a dikaryotic phase (Glass and Nelson, 1994 and Kendrick, 1992).

The Ascomycota is distinguished from the Basidiomycota by the production of meiospores (ascospores) formed within sac-shaped cells (asci) which may or may not be produced within a sporocarp (ascoma). Hence, they are commonly known as sac-fungi (Blackwell and Spatafora, 2004).

1.3.1 Classification of Ascomycota

Ascomycetes are divided into three monophyletic subphyla (Lutzoni amd Kauff, 2004; James *et al.* 2006 and Hibbett *et al.* 2007):-

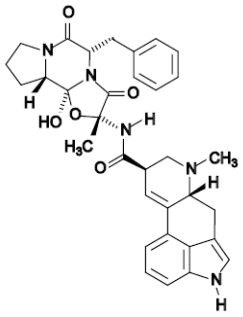
Subphylum *Taphrinomycotina* (formerly *Archiascomycetes*) is resolved as the earliest diverging clade; it includes a diverse group of species that exhibit yeast-like and dimorphic forms.

Subphylum *Saccharomycotina* (formerly *Hemiascomycetes*) is comprised of the true yeasts such as *Saccharomyces cerevisiae* and *Candida albicans*. Subphylum *Pezizomycotina* (formerly *Euascomycetes*) is the largest subphylum comprising the majority of species that produce hyphae and ascocarps (fruiting bodies). All of the fungi presented in this thesis are members of this subphylum.

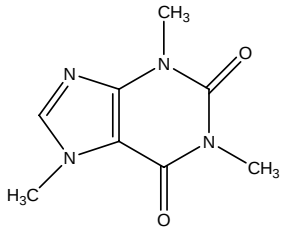
1.3.2 Ecology and significance of Ascomycetes

Some of the Ascomycota are known to be pathogenic causing diseases to plants, animals and humans. Some of the most common infections to humans are dandruff and athlete's foot. Some fungi, however, can cause far more devastating diseases which have very high associated mortality rates such as, invasive aspergillosis (caused by *Aspergillus* sp.), candidosis (caused by *Candida* sp.) and Pneumocystic pneumonia (caused by *Pneumocystis jiroveci*.) (Sullivan *et al.* 2005). Furthermore, many plant diseases have been noted that are caused by Ascomycetes. For example, powdery mildew fungi of many crop plants, Dutch Elm Disease, (caused by the closely related species *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*) and Ergot (caused by *Claviceps purpurea*) (Deacon, 2006). Nevertheless, Ascomycota have been utilized in the food processing and manufacturing world. For example, baker's yeast *Saccharomyces cerivisiae* is used to make bread, beer and wine. Another example is the enzyme of *Penicillium* species, the enzyme from *Penicillium camemberti* played a role in the manufacture of the cheeses Camembert and Brie, while those of *Penicillium roqueforti* do

the same for Gorgonzola, Roquefort and Stilton cheese. Apart from the food processing aspect, they also play a role in the production of valuable commodities such as antibiotics, enzymes, vitamins and pharmaceutical compounds (Murphy and Horgan, 2005). A classic example is the discovery of penicillin in 1928. Another example of a commercial medicine isolated from Ascomycota is that of the ergot alkaloids and related toxins of the ergot fungus, *Claviceps purpurea*, which have many important pharmacological applications. An example includes the antimigraine drug "cafergot" which consists of ergotamine and caffeine **(Figure 1.11**) Diener *et al*. (2002).



ergotamine



caffeine

Figure 1.11: Ergotamine and caffeine

Various types of antifungal agents are also produced by Ascomycota. For example, a glucan synthesis inhibitor such as echinocandin B (**Figure 1.12**) was produced by *Aspergillus nidulans.* Protein synthesis inhibitors such as sodarin (**Figure 1.13**) and xylarin (**Figure 1.14**) were derived from *Sordaria araneosa* and *Xylaria* sp. respectively.

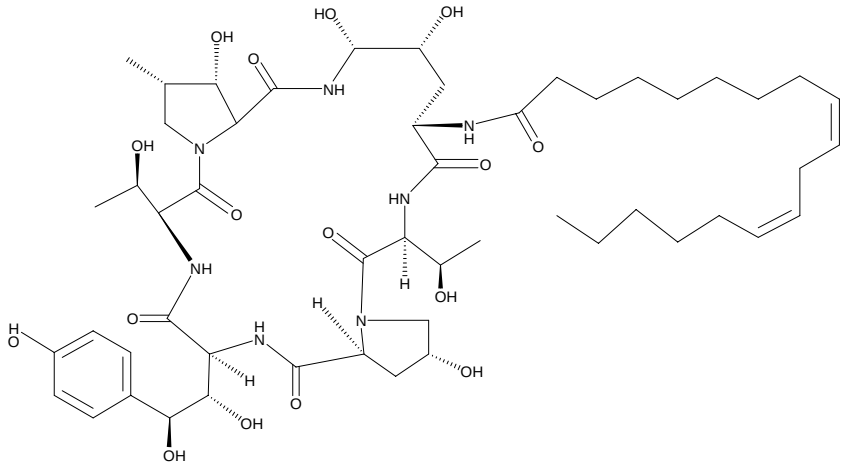


Figure 1.12 Echinocandin B

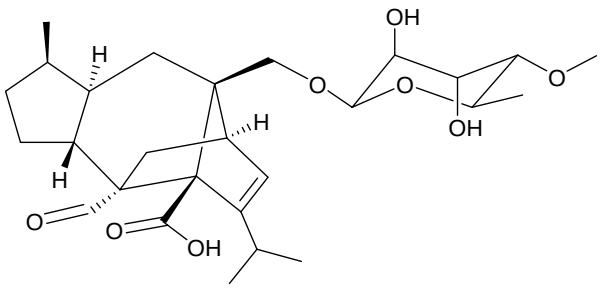


Figure 1.13 Sordarin

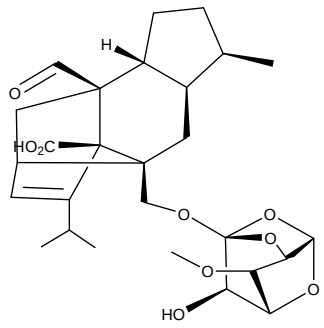


Figure 1.14 Xylarin

1.4 SELECTIVE ISOLATION TECHNIQUES

In the past, routine soil sampling has yielded many natural products, but often these have been obtained from easily accessible and culturable organisms while the lesser, or non-culturable variants remains an untapped resource. One reason for this is that the isolation strategies employed have not allowed the less culturable organisms to compete with the more easy accessible and culturable organisms. Moreover, most isolation media allows for the growth of only a selected group of strains and inhibits the majority of the natural population (Knight *et al.* 2003). Undoubtedly, isolation strategies for the less readily culturable organisms would enhance the potential for the discovery of novel biotypes and new bioactive compounds.

1.4.1 Isolation techniques for soil fungi

There are various methods used to isolate fungi from soil. The standard methods are the 'suspension plating method' and the 'soil dilution method'. These methods are simple and rapid, but yield a large number of fungal isolates. Another method which has been used in the past and still widely used today is the 'Warcup soil plate method'.

In this method finely pulverized soils are dispersed into a sterile Petri dish. Molten agar is poured over the soils and then quick, but gentle, agitation of the plates ensures that the soil is mixed with the agar thoroughly. The yield of this method is similar to the suspension plating method, but may also

yield basidiomycetes which are not recovered by the suspension plating method (Warcup, 1950). One disadvantage of the Warcup soil plate method is that young colonies which are embedded in agar are difficult to isolate. Fungi derived using the methods described above originate either from spores, or mycelial fragments. Mycelial derived isolates can be enhanced by using a particle filtration technique (Gerald *et al*. 2004).

All of the above methods rapidly produce large numbers of isolates over a wide range of species that grow readily in culture. Unfortunately, high propagule densities of fast growing fungi can interfere with the isolation of the rarer and/or slow growing species (Gerald *et al*. 2004).

1.4.2 Selective isolation methods for soil fungi

Many isolation methods have been introduced for the selection of certain groups of fungi. The baiting technique is such a method. In this technique an organic substratum is enriched nutritionally and then placed in contact or mixed with soil thought to contain target organism(s). This method can be biased towards animal or plant pathogenic fungi. For example, using cellulose baits tends to be selective for plant pathogenic fungi; keratin baits for animal pathogenic fungi and chitin baits are more selective for entomopathogenic fungi.

Several partial pasteurization methods using either physical pasteurization factors such as dry heat or steam, or chemical treatments, either singly or in combination have been applied for selective recovery of ascomycetes and basidiomycetes from soil habitats (Bills and Polishook, 1993).

During the 1940s and 1950s studies on chemical activation of dormant ascospores were conducted in conjunction with selective isolation methods for ascomycetes. Warcup (1951[a]) proposed that short steam treatment or an alcohol wash favoured isolation ascosporic Eurotiales, Sordariales and other ascomycetes from soils. The effectiveness of these methods was confirmed by Mahoney (1972). He also found that these treatments decreased the frequency of isolation of mucoraceous fungi and fungi with thin-walled propagules and increased the population of ascosporic fungi. Many chemical compounds have been studied for their influence on ascospore germination. For example, sodium acetate was used by Asina *et al.* (1977) to induce the ascospore germination of *Sporomiella sp.*

In 1970 Lingappa *et al.* developed a phenol technique to activate ascospore germination. Weber and Hess (1976) also used a phenol technique to study the physiology of constitutive dormancy and germination of ascospores. A more detailed of study was carried out by Furuya and Naito (1980) on stimulation of ascospore germination by phenolic compounds. In total 36 Phenol derivatives and 26 organic compounds were tested on 37 fungal species. In their study, two phenol concentrations were used, (1×10^{-3} M and 1×10^{-4} M), and 14 out of the 37 fungal species were found to respond to phenol within this concentration range. Moreover, the stimulating effect of phenol was restricted to sordariaceous fungi. For phenol derivatives and other organic compounds the results showed that 10 out of 62 compounds had an effect on ascospore germination. Of those 10 compounds, one was phenol and the rest were *para*-substituted phenol derivatives. However, phenol was shown to be the most effective in activating many dormant

ascospores to germinate. In this study a modified phenol pasteurization technique was used based on the acetic acid treatment method of Furuya and Naito (1979[a]) in an attempt to select Ascomycetes from New Zealand soils that would not normally be isolated using other methods.

1.5 MANIPULATION OF THE FUNGAL METABOLITE PRODUCTIONS

Apart from the attempt to access the biodiversity and explore untapped natural resources for discovering novel bioactive compound, another option is to elevate metabolite production by influencing the biosynthetic pathways for the production of secondary metabolites. It is believed that secondary metabolite production is affected by various kind of environmental stress. Even minor variations in the environment or nutrition have the potential to impact on the quantity and diversity of the secondary metabolites produced (Bills *et al.* 2008; Zain *et al.* 2009).

*1.5.1 **O**ne **S**train **Ma**ny **C**ompound (OSMAC) approach*

Bode *et al.* (2002) stated that fungi or bacteria that produce secondary metabolites have the potential to biosynthesise a wide variety of compounds from a single strain. A systematic alteration of cultivation parameters such as media composition, aeration, temperature, culture vessel, or addition of enzyme inhibitors could possibly increase the range and number of secondary metabolites produced by one microbial source. This strategy is termed the **O**ne **S**train-**Ma**ny **C**ompounds (OSMAC) approach. This approach has been applied in many recent studies (Bode *et al*. 2000[a]). Simplest work using the OSMAC approach was carried out by Paranagama *et al.* (2007). In this study the change from tap water to distilled water showed an alteration of metabolite productions in *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii*. An alteration of the fermentation conditions also has an effect the metabolite productions in fungi, Bode *et al*. (2000[a]) showed that Sphaeropsidales species produce more metabolites simply by changing the culture condition from a shaking fermentation to a static fermentation. Another example of an OSMAC approach is by the addition of organic and/or inorganic compounds into the media. This approach also induced secondary metabolite production in *Streptomyces* sp. (Grond *et al.* 2002).

1.5.2 Hormesis

As a result of the many studies conducted it has been concluded that a modulation in cell transcription occurs in the presence of antibiotics or inhibitors at low concentration. In this situation they act as signalling rather than inhibiting agents (Hoffman *et al.* 2005; Linares *et al.* 2006 and Yim *et al.* 2006). The use of subinhibitory concentrations of these elicitors is based on the concept of hormesis which was defined as low-dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2002). This is an adaptive response of organisms to low levels of stress or damage. The use of elicitors such as antibiotics and cytoskeleton inhibitors at subinhibitory concentrations has been shown to alter secondary metabolite production in actinomycetes, bacteria and fungi. For example, the presence of tetracycline in the media was found to induce phenazine production in a marine *Streptomyces* sp. (Mitova *et al*. 2008[a]). The use of an actin stabilizer such as jasplakinolide was found to induce the production of chaetoglobosin in *Phomospis asparagi* (Christian *et. al*. 2005). The intention of the studies carried out in this thesis were to explore and studiy the OSMAC approach and hormesis in greater detail.

1.6 SCOPE OF PRESENT STUDY

The main goals of the present study were:-

1) The development of a selective isolation technique for less readily isolatable fungi from soil, especial Ascomycetes. The phenol pasteurization technique was used as a selective isolation technique for Ascomycetes in soil samples collected from various locations around New Zealand.
2) Chemical characterization of metabolites from fungal extracts. Small scale cultivation of isolates of fungi was undertaken. The fungal extracts were screened for biological activity such as cytotoxicity and antimicrobial activity. The chemical separation in active extracts was aided by HPLC. Chemical characterization of metabolites of after separation active extracts was greatly aided by dereplication techniques whereby a combination of HPLC-UV/R_t database, MS analysis, the CapNMR technique and AntiMarin database were used.
3) Maximize metabolic production and induce the production of metabolites. The effect of culture conditions (both physical and chemical) were undertaken based on the OSMAC (**O**ne **S**train **Ma**ny **C**ompounds) and hormetic (low-dose stimulation and high-dose inhibition) approach.

Chapter 2

Experimental

2.1 ORIGIN OF SOIL SAMPLES

Soil samples from various locations in New Zealand were collected by Mr. Craig Galilee and Nic Cummings of the School of Biological Sciences, University of Canterbury. Eleven randomly selected soil samples were used for the isolation of fungi using the phenol treatment method as shown in **Table 2.1**.

Table 2.1: Origin of soil samples

Soil codes	Location	Date of isolation
151	Fox Glacier (submerged soil)	13-Jan-2006
381	Arthurs Pass (stream edge)	13-Jan-2005
418	Bay of Plenty (inside cotton log frass)	24-Jan-2006
456	Bay of Plenty (river edge soil)	31-Jan-2006
654	Nelson	31-Jan-2006
701	Tasman walk site 1	24-Jan-2006
702	Tasman walk site2	19-Jan-2006
770	Eves Bush, Nelson (inside cotton tree stump)	19-Jan-2006
901	Tiromoana Bush site 1	05-Oct-2007
902	Tiromoana Bush site 2	05-Oct-2007
903	Tiromoana Bush site 3	05-Oct-2007

2.2 ISOLATION AND CULTIVATION MEDIA

Malt Yeast Peptone Agar (MYPA) and ¼ strength of Sabouraud Dextrose Agar

(¼ SDA) were used for the isolation and cultivation of the fungi, see **Appendix I**.

2.3 PHENOL PASTEURIZATION OF SOIL

Method

- 5.0 g of soil sample was placed in each of four sterile 125 mL conical-flasks labeled; 0, 2, 3, 4, 5, 6, 7, 8.
- Soil in flask labeled 0 was treated with 20 mL sterile distilled water (control). Soils in flasks 2, 3, 4, 5, 6, 7, 8 were treated with 20 mL of 2%, 3%, 4%, 5%, 6%, 7% and 8% phenol solution in sterile distilled water respectively.
- Flasks were agitated on a reciprocal shaker for five minutes and followed by 15 minutes settling time.
- The supernatant was discarded and the soil rinsed twice with 5 mL sterile distilled water, and then 5 mL sterile distilled water was added to the washed soil to make a soil suspension.
- Treated soil suspension (0.7 mL) was pipetted into an 85 mm Petri dish. 15 mL of Sabouraud Dextrose Agar plus antibiotic solution

(SDA^{+}) (section 2.3) was dispensed over the suspension. Seven replicate plates were prepared for each sample.

- Each plate was agitated gently in order to mix the suspension with the molten agar.
- After the agar had set, the plates were incubated at; 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C in the darkness. A flow-chart of the method used is given in **Figure 2.1**.

For the control (0% phenol treatment without soil suspension), 0.7 mL sterile distilled water was pipetted into an 85 mm Petri dish and mixed with 15 mL of SDA^{+}; seven replica plates were prepared. When the agar solidified, these plates were also incubated at; 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C, in darkness.

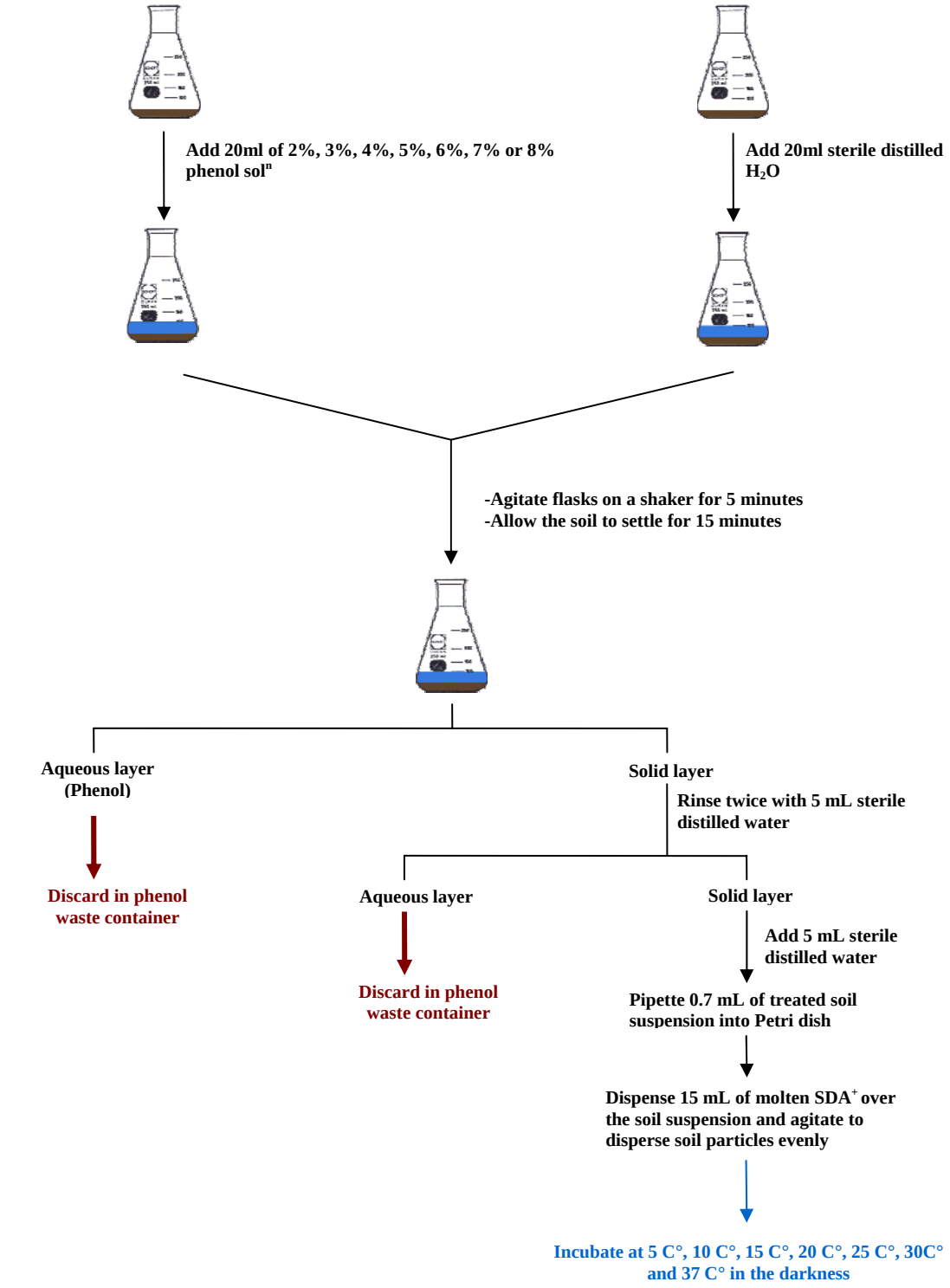


Figure 2.1: Phenol pasteurization method

2.4 CULTURING METHODS

2.4.1 *Selection and culture of fungi*

Plates were observed for colonies at 3, 7, 14, 21 and 28 day intervals. All colonies present on the master plates were sub-cultured onto 85 mm Petri dishes containing 20 mL of SDA^{+} and incubated as indicated previously. Colonies from 7, 14, 21 and 28 day incubation plates were chosen for sub-culturing to SDA plates to obtain axenic cultures.

2.4.2 *Storage of fungal cultures*

Discs (4 mm in diameter) were cut from the growing margin of seven day old fungal colonies grown on malt-yeast-peptone agar plates (MYPA; **Appendix I**). Seven discs were placed into a 1.5 mL microfuge tube containing 10% glycerol in distilled water and stored overnight at -20 °C before transferred to -80 °C for long-term storage.

2.4.3 *Fermentation methods*

2.4.3.1 Solid phase fermentation

Seven day pure cultures from growth at 20 °C, 25 °C, 30 °C or 37 °C were selected for solid phase fermentation. Inoculum was prepared by cutting agar discs, 4 mm in diameter, from the margin of a colony. The inoculum disc was inverted onto the centre of an 85 mm Petri dish containing 20 mL of MYPA.

Inoculated plates were incubated under the same conditions as their master plate for 30 days. For each isolate, three replicate experiments were carried out.

2.4.3.2 Liquid phase fermentation

Two broth media were used; ¼ Sabouraud dextrose broth (SDB; **Appendix I**) and Malt Yeast Peptone Broth (MYPB; **Appendix I**). Medium (15 mL) was dispensed in universal bottles and inoculated with two inoculum discs. The universal bottles were either held static or shaken on a reciprocal shaker at 125 rpm, or a mixture of shake/static (four replicates per condition). For the shake/static incubation, the first half of the incubation period was shaken as outlined previously and the second half of the incubation period was kept static. Incubation was carried out for 20 days and 30 days at 20 °C and 25 °C in the darkness. A summary of the liquid phase fermentation conditions is given in **Table 2.2**.

Table 2.2: Liquid phase fermentation conditions

Media	Temp (°C)	Inoculation period (Day)	Conditions	replicates
MYPB	20	20	static	4
			shaken	4
			½ shaken ½ static	4
		30	static	4
			shaken	4
			½ shaken ½ static	4
	25	20	static	4
			shaken	4
			½ shaken ½ static	4
		30	static	4
			shaken	4
			½ shaken ½ static	4
SDB	20	20	static	4
			shaken	4
			½ shaken ½ static	4
		30	static	4
			shaken	4
			½ shaken ½ static	4
	25	20	static	4
			shaken	4
			½ shaken ½ static	4
		30	static	4
			shaken	4
			½ shaken ½ static	4

2.5 IDENTIFICATION OF FUNGI

Colonies of isolated fungi were examined under a stereoscopic microscope and morphological features of the mycelium and reproductive structures examined using a compound microscope with magnification up to 400×. Higher resolution images of these features were obtained using a scanning electron microscope (SEM). Fungi were identified in consultation with Assoc. Prof. Tony Cole and Dr. Nic Cummings.

For the scanning electron microscope specimen preparation, a mounted stub with double sided sticky tape was plunged into the culture area bearing reproductive structures. The specimen was then coated with a thin coating of gold palladium (Au-Pt) using a Polaron E5000 coater with the following conditions: 1.2 KV at 20 mA for 2 minutes. Micrographs were taken by a Lieca S440 at 10 kV and 80 pA. The working distance was approximately 10 mm.

2.6 PREPARATION OF CULTURE EXTRACTS

2.6.1. *Solid phase extraction*

Agar from plates with fungal cultures were macerated with 30 mL HPLC grade ethyl acetate (EtOAc) using an Ultra-turrax®. This extraction process was carried out three times. The agar slurry was left sitting overnight for the first EtOAc extraction, and eight hours each for the second and third extractions. All three EtOAc extracts were combined and transferred to a pre-weighed vial and

air dried to yield a crude extract. Crude extracts were prepared to a concentration of 1 mg/mL in HPLC grade methanol for bioassays. The procedure for the solid phase extraction process is summarised in **Figure 2.2**.

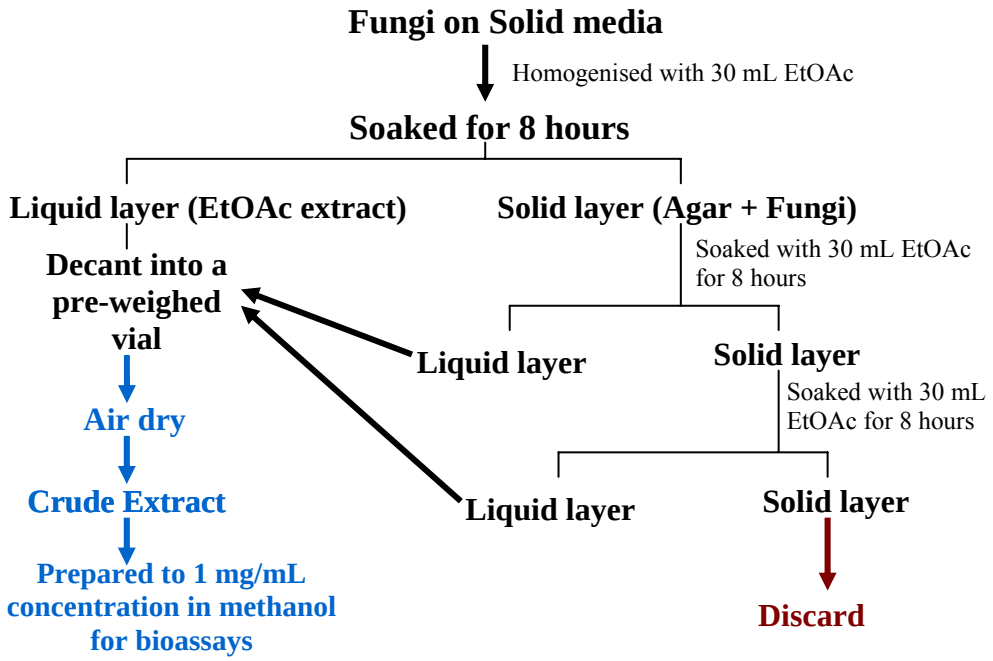


Figure 2.2: Solid phase extraction method

2.6.2. *Liquid phase extraction*

Two universal bottle cultures were combined and transferred into a 50 mL Pyrex® centrifuge tube with a round bottom and homogenised using an Ultra-turrax® for three minutes, or until the inoculum agar discs and mycelial mat were sufficiently macerated. The mixture was filtered through Whatman® filter paper grade No. 1 under suction to separate mycelium and broth. The filtrate broth was subsequently extracted three times with EtOAc at a ratio of 3:1 (30 mL EtOAc per 10 mL filtrate). The mycelial residue was re-suspended in EtOAc at a ratio of 1:5 (100 mL EtOAc per 20 g wet weight mycelium) and extracted three times. The initial EtOAc extraction was left to settle overnight

and the second and third for eight hours. All organic phases were combined and air dried to yield a crude extract. Crude extracts were prepared to 1 mg/mL concentration in methanol for further bioassays.

2.7 BIOASSAYS

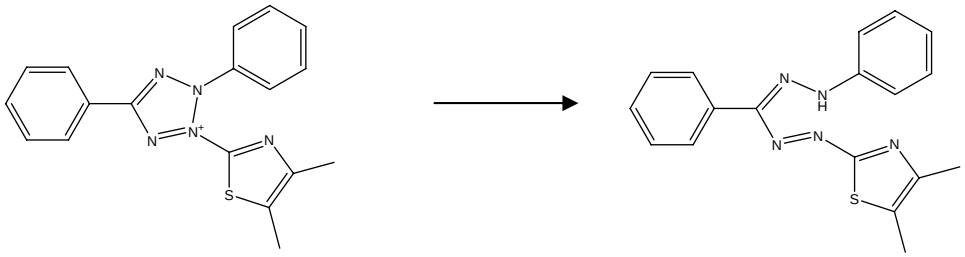
Extracts were initially screened in a preliminary quick screen assay. Crude extracts (1 mg/mL concentration in HPLC grade methanol) were used for quick screen bioassays. An aliquot of each extract was pipetted into individual wells of a 96-well microtitre plate. For the cytotoxicity quick screen, 5 µL per sample was assayed against the murine leukaemia cell line P388 (ATCC CCL 46, P388D1). For the antimicrobial quick screens, 10 µL per sample were used to test against *Bacillus subtilis* and *Pseudomonas aeruginosa*, while 20 µL per sample was used to test against *Candida albicans.*

2.7.1 *Cytotoxicity (P388) assay*

2.7.1.1 P388 Quick screen assay

Crude extracts were first subjected to the quick screen assay. In this assay, P388 cells (8.4×10^4 cells/mL) were incubated with each crude extract for 72 h at 36 °C. Cell viability was determined colorimetrically by the addition of a yellow dye, MTT tetrazolium. Unhealthy or dead cells cannot metabolise this dye, thereby, leaving a yellow colour. Healthy cells are able to reduce the dye to MTT formazan resulting in an intense purple colour (**Figure 2.3**). The UV

absorption of each sample was measured at 540 nm and compared with the UV absorption of a control cell. Results were expressed as the percentage of cell growth compared to normal cell growth. Those samples showing good activity in the P388 quick screen (<20% cell viability) were subsequently subjected to a further assay to determine the concentration required to inhibit the growth of P388 cells by 50% (IC_{50}) (Perry *et al*. 1999). Samples were also separated and analysed by high pressure liquid chromatography (HPLC).



MTT tetrazolium salt (yellow) MTT formazan (purple)

Figure 2.3: Reduction of the yellow MTT fetrazolium salt to the purple MTT formazan by health P388 cells.

2.7.1.2 IC_{50} assay

Active crude extracts were prepared at a concentration of 10 mg/mL in HPLC grade methanol and assayed. This assay requires a serial dilution of the sample of interest followed by incubation for 72 h with P388 cells (**Figure 2.4**). The growth indicator was the same as that used in the P388 quick screen assay. The result is expressed as an IC_{50} in ng/mL.

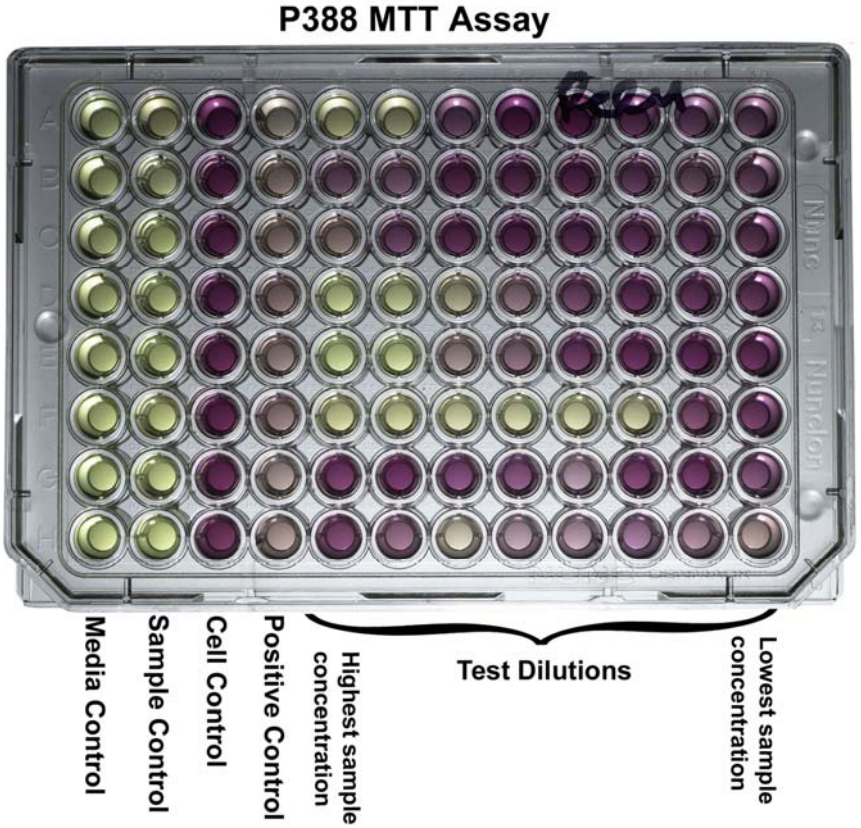


Figure 2.4: P388 assay dilution plate: purple indicates viable cells and yellow indicates dead or unhealthy cells.

2.7.2 Antimicrobial assay

Bacterial and fungal suspensions were prepared in Roswell Park Memorial Institute-1640 medium (RPMI-1640). The density of cells was measured at 600 nm and adjusted to an optical density (OD) of 0.04 for *B. subtilis*, and 0.05 OD for *P. aeruginosa* and *C. albicans*. 200 µL of the bacterial or fungal suspension was incubated with each crude extract for 24 h at 30 °C . The cell viability was determined colorimetrically by the addition of the blue dye, resazurin. Resazurin is a growth indicator and the presence of bacteria or fungi

is indicated by oxygen emission which reacts with resazurin, generating a pink colour. The UV absorption of each sample was read at 600 nm and compared against the UV absorption of the control media. Results are given as a percentage of cell viability (Lang *et al*. 2006). Crude extracts which had a result less than 10%; i.e. 90% of bacterial or fungal cells were killed by the crude extract, were noted and analysed by HPLC.

2.7.3 HPLC microtitre plate screening

An aliquot of the crude extract (250 µL) was analysed by reversed phase C_{18} HPLC (solvents (A) H_2O + 0.05% formic acid, (B) ACN) using the following standard gradient solvent system: 2 minutes of 10% ACN/H_2O; a linear gradient to 75% ACN/H_2O for 12 minutes; isocratic at 75% for another 10 minutes; a linear gradient to 100% ACN for 4 minutes then returned to 10% ACN/H_2O over 2 minutes and re-equilibrated for 8 minutes. The flow rate was 1 mL/min at 40 °C. The eluant from the HPLC column was collected into a master plate, 96-well polystyrene microtritre plate, with a total of 88 wells being collected and each well containing a total of 250 µL. A daughter plate was prepared by transferring 50 µL aliquots from each well of the master plate. After complete evaporation of the solvent, using a centrifugal evaporator, the plates were screened in the P388 assay. In the antimicrobial screens, the master plate was used directly after evaporation of the solvent. No daughter plates were made up.

2.8 ISOLATION AND IDENTIFICATION OF ACTIVE COMPOUNDS

As part of the isolation and identification process all small scale extracts were initially examined by HPLC to separate the compounds in the crude extracts. Mass spectrometry (MS) analysis was carried out to attempt to determine molecular mass of each compound. Pure compounds were subjected to Nuclear Magnetic Resonance (NMR) spectroscopy for the structure elucidation.

2.8.1 HPLC screening of metabolites

Crude extracts were prepared at a concentration of 1 mg/mL in HPLC grade methanol. All samples were filtered through 0.45 µm polytetrafluoroethylene (PTFE) membrane filters immediately prior to injection. An aliquot of the crude extract (30 µg) was analysed by analytical HPLC on a reverseed phase column (Phenomenex Luna C18, 10×250 mm, 5µm; Solvents: (A) H_2O + 0.05% TFA, (B) ACN) using the standard gradient solvent system (see **Section 2.7.3**).

2.8.8.1 Separation of extract F9330

All other extracts were screened using the standard gradient (see **Section 2.7.3)** except extract F9330 as in this case the metabolite peaks, which eluted over the period R_t 8.0-10.0 min, were not separated by the standard gradient. A 25-40% gradient was developed for this particular extract: 2 minutes of 10% ACN/H_2O; a linear gradient to 20% ACN/H_2O for 3 minutes; a linear gradient to 25% ACN/H_2O for 7 min; a linear gradient to 40% ACN/H_2O for 8 min; a linear gradient to 75% ACN/H_2O for 2 min; isocratic at 75% for another 10 minutes; a linear gradient to 100% ACN for 4 minutes then returned to 10% ACN/H_2O over 2 minutes and re-equilibrated for 8 minutes.

2.8.2 *Electrospray ionization mass spectrometry (ESIMS)*

High Resolution Electrospray Ionization Mass Spectra (HRESIMS) were obtained on a Kratos MS80RFA spectrometer, operating with a 4 kV accelerating potential, 70 eV and a source temperature of 250 °C. HRESIMS were recorded on a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150 °C and a source temperature of 80 °C. The carrier solvent was 50:50 ACN/H_2O at 20 µL/min (for direct inject mode). A 10 µL injection of sample was made from a 10 µg/mL stock solution. When obtaining positive ESI mass spectra, some samples were protonated with 10 µg/mL formic acid prior to injection. When recording negative ESI mass spectra, samples were deprotonated, as required, with 10 µg/mL DEA prior to injection.

2.8.3 Liquid chromatography mass spectrometry (LCMS)

High Resolution Liquid Chromatography Mass Spectra (HRLCMS) were recorded on a Waters 2790 HPLC system equipped with a Waters 996 photodiode array (PDA) detector coupled to a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150 °C and a source temperature of 80 °C. The carrier solvent was 50:50 ACN/H_2O at 20 µL/min (for direct inject mode). A 10 µL of a 10 µg/mL solution was injected. Leucine enkephalin was used as the internal standard.

2.8.4 Nuclear magnetic resonance (NMR) spectroscopy

Samples used in this analysis were obtained from analytical HPLC microtitre plate collection using the standard method given in **Section 2.7.3** except that the eluant was collected into a polypropylene microtitre plate instead of the polystyrene microtitre plate. Master plates were dried using centrifugal evaporation; no daughter plates were made. ^{1}H, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation spectroscopy (HMBC) and nuclear overhuser effect (NOE) NMR experiments were all recorded on a Varian INOVA 500 spectrometer at 23 °C, operating at 500 MHz. The INOVA was equipped with a variable temperature and capillary NMR probe (**Figure 2.4**). Chemical shifts are expressed in part per million (ppm) on the δ scale, and were referenced to the appropriated solvent peaks:

$CDCl_3$ referenced to C**H**Cl_3 at δ_H 7.25 ppm (^1H) and **C**HCl_3 at δ_C 77.0 ppm (^{13}C);
CD_3OD referenced to C**H**D_2OD at δ_H 3.30 ppm (^1H) and **C**HD_2OD at δ_C 49.3 ppm (^{13}C);
DMSO-d_6 referenced to $CD_3(CHD_2)SO$ at δ_H 2.50 ppm (^1H) and $(CD_3)_2SO$ at δ_C 39.6 ppm (^{13}C).

Figure 2.5: Protasis capillary NMR probe.

The pure compound obtained from the method described in **Section 2.7.3** was dissolved in 6 µL of CD_3OD and injected into an inlet capillary via a syringe. This was then followed by an injection of 11 µL of deuterated MeOH to position the sample in the probe. All samples were retrieved into HPLC vials or inserts that were silated as described in **Section 2.8.5**. The quantity of the compound in each well (A) was estimated according to the formula below.

$$A\ (\mu g) = \frac{\text{Molecular weight of A}}{\sum \text{protons of A}} \times \frac{\sum \text{protons (solvent – A)}}{\sum \text{protons (solvent)}} \times 0.35$$

2.8.5 Silanization of glassware surface for CapNMR

Due to the low concentration of sample analysed in CapNMR, all glassware used in CapNMR analyses were deactivated the surface to minimize adsorption in sample. All glassware was immersed into dimethyldichlorosilane (DMDCS) solution (5% DMDCS in dichloromethane (DCM)) for 15 min. After pouring off the deactivating solution the glassware was immediately rinsed with DCM and then immediately immersed in MeOH for 15-30 mins. This step was performed immediately in order to minimize exposure to moisture in the air. The MeOH was drained off and the glassware was dried in a 100 °C oven until completely dry. The DMDCS solution was freshly prepared before use.

2.8.6 Solvents

All solvents used in the chromatographic techniques were HPLC grade. Analytical grade solvents were used for other analyses.

Chapter 3

Development of a selective isolation technique for Ascomycetes

3.1 INTRODUCTION

Several isolation techniques have been developed and employed for isolating slow-growing fungi and eliminating fast-growing fungi. These include physical treatments. Steam treatment has been found to be an effective method for isolation of Ascomycetes (Warcup, 1951[a]), however, it does not favour the isolation of *Chaetomium* sp. or Sordariaceae (Warcup, 1951[a]). Furthermore, many studies have reported that the soil-steaming method only gives an initial depression of the growth of Mucorales and does not eliminate them permanently (Tam and Clark, 1943 and Martin, 1950).

Various chemical agents have also been used in partial sterilization and some disadvantages have been reported. Formalin was found to be a very effective agent to eliminate fast-growing fungi, but the growth of fungi on the isolation plates is very slow and time consuming (Warcup, 1951[b]). Ethanol is another agent that has been used in a partial sterilization method

(Bills and Polishook, 1993) but resulted in a lot of sterile strains rather than ascosporic fungi being isolated. In the study by Furuya and Naito (1979[b]), ethanol was used as a selective isolation agent with the addition of sodium acetate as an ascospore germination stimulator. The effectiveness of the use of ethanol as a selective isolation method was therefore somewhat confused. Acetic acid, which was shown to stimulate ascospore germination (Butler, 1956) was found to be effective for the isolation of Ascomycete genera such as *Chaetomium*, *Eupenicillium*, *Gelasinospora*, *Sordaria* and *Talaromyces* (Furuya and Naito, 1979[a]). This study showed similar results to the present phenol study.

3.2 ISOLATION OF FUNGI

3.1.1 *Colony growth after phenol pasteurization*

All 11 soil samples were treated with a range of phenol concentrations (2% to 8%) and plated out as described in **Chapter 2**. Colonies were counted and recorded after 3, 7, 14, 21 and 28 days incubation see (**Table I-XI, Appendix II**). Average colony numbers after 3, 7 and 14 days incubation for these 11 soil samples after phenol treatment are given in **Figures 3.1-3.3.**

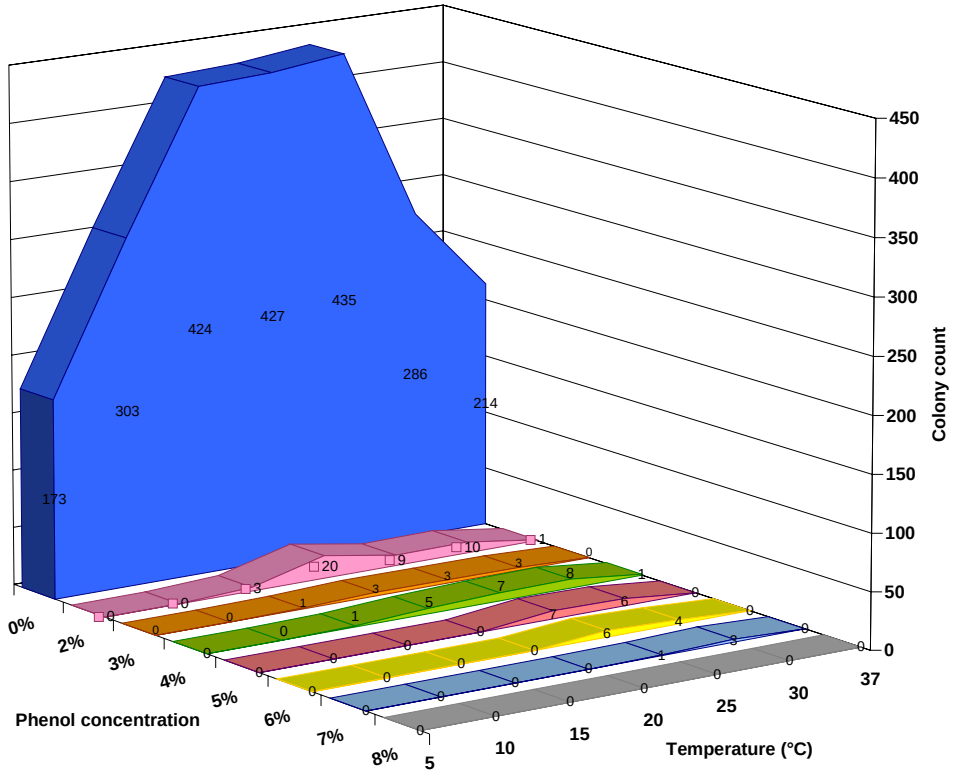


Figure 3.1: Average colony numbers at 3 days

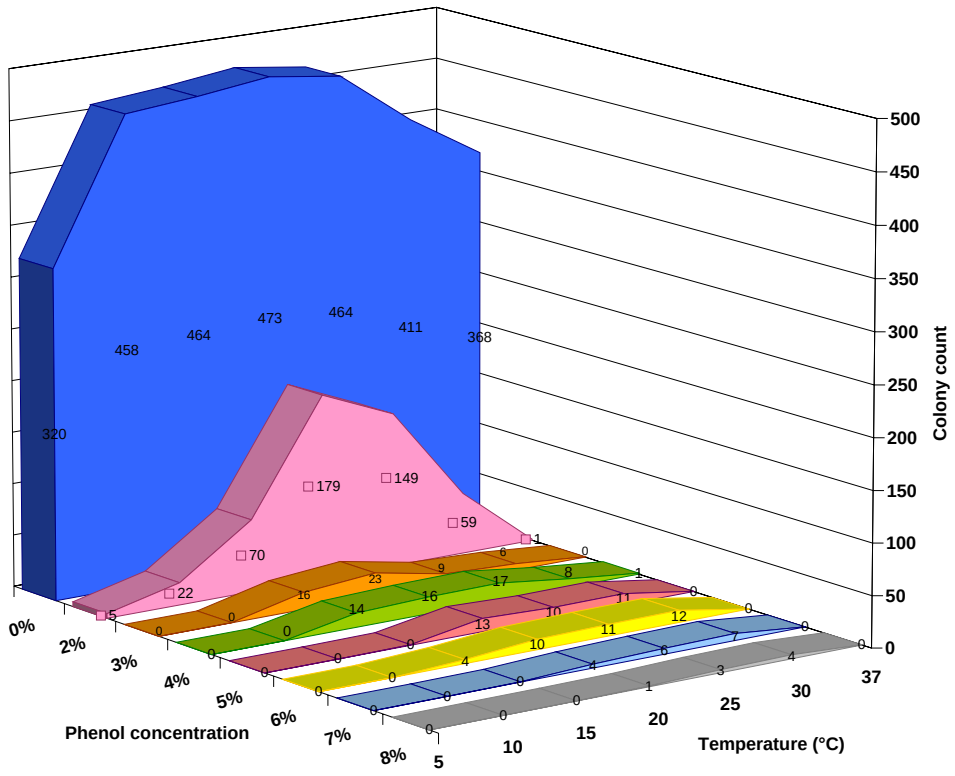


Figure 3.2: Average colony numbers at 7 days

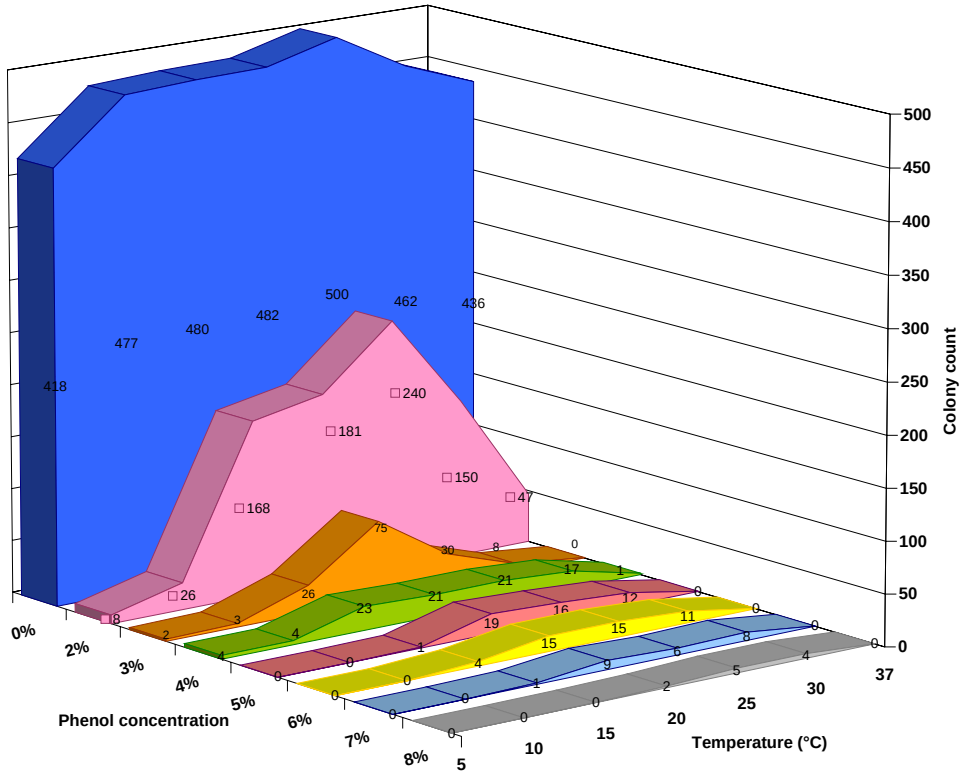


Figure 3.3: Average colony numbers at 14 days

After 3 days incubation the untreated soil plates (0% phenol treatment) were overrun by mucoraceous fungi and no ascomycetes could be distinguished. At this point, soils treated with 2-8% phenol showed no sign of mucoraceous fungi. However, it was seen that after 7 days incubation some plates from soils treated with 2% phenol solution were overrun by mucoraceous fungi, but not at any higher phenol concentrations. After 14 days incubation some plates from soil treated with 3% phenol were overrun by mucoraceous fungi, but not with the soils treated with 4% phenol and above. Furthermore, those soils treated with 4% phenol and above showed no sign of mucoraceous fungi being present at the end of the 28 day observation period.

Apart from phenol concentration, temperature also showed a profound effect on the growth of the fungi. **Figures 3.1- 3.3** clearly show that the optimum temperature for growth was 20 °C followed by 25 °C, with little growth occurring at 5 °C or 37 °C.

A comparison of the average colony numbers of the three day (**Figure 3.1**) and seven day incubation (**Figure 3.2)** showed a dramatic increase in the average colony numbers observable with treatment both at 20 °C and 25 °C. This was, however, a result of the growth of mucoraceous fungi. This also accounted for the large increase of colony numbers after 14 days (**Figure 3.3**) with 2% phenol treatment at 25 °C and with the 3% phenol treatment at 20 °C.

3.1.2 Identification of fungal isolates

Untreated plates mainly yielded fast growing mucoraceous species. With 2% phenol treatment, most of the fungi that were recovered were sterile or anamorphic fungi including *Aspergillus* sp., *Aureobasidium* sp., Cephalosporium sp., *Fusarium* sp., *Gliocladium* sp., *Isaria farinosa*, four different strains of *Penicillium* and *Verticillium tenerum*. Several ascosporic fungi were also recovered from this treatment and identified as *Gymnoascus* sp. and *Talaromyces* sp. With 3% phenol treatment and above, a few sterile and anamorphic fungi were recovered including *Aureobasidium* sp., *Gliocladium* sp., *Humicola* sp., *Penicillium* sp. and *Verticillium tenerum*,

however, the majority of species were ascomyceteous including *Chaetomium globosum*, *C. trigonosporum*, *Eupenicillium* sp., *Eurotium* sp., *Gymnoascus* sp., *Melanospora* sp., *Sodaria* sp., *Spherodes* sp., *Talaromyces* sp., *Thielavia* sp. and *Xylaria* sp.

A total of 407 isolates were obtained. The number of fungal isolates obtained from each phenol concentration is shown in **Table 3.1**.

Table 3.1: Fungal isolates obtained from soil samples treated with different phenol concentrations.

Phenol concentration	Isolation medium	Number of isolates
2%	MYPA	129
3%	MYPA	101
4%	MYPA	82
5%	MYPA	55
6%	MYPA	19
7%	MYPA	13
8%	MYPA	8
Total		**407**

Isolates were initially identified to the genus level where possible based on their morphology and colony characteristics. A number were able to be identified to the species level by their specific characteristics. A summary of identified fungal isolates obtained from each phenol treatment is shown in **Table 3.2**.

Table 3.2: Identified fungal isolates from different phenol treatments.

Phenol 2%	**Phenol 3%**	**Phenol 4%**	**Phenol 5%**	**Phenol 6%**	**Phenol 7%**	**Phenol 8%**
Aspergillus sp.	*Aspergillus* sp.	*Bombardia* sp.	*Aureobasidium* sp.	*Cephalosporium* sp.	*Aureobasidium* sp.	*Cephalosporium* sp.
Aureobasidum sp.	*Chaetomidium* sp.	*Chaetomium globosum*	*Chaetomium globosum*	*Eurotium* sp.	*Gymnoascus* sp.	
Cephalosporium sp.	*Chaetomium globosum*	*Chaetomium trigonosporum*	*Eupenicillium* sp.	*Thielavia* sp.	*Talaromyces* sp.	
Fusarium sp.	*Chaetomium trigonosporum*	*Eupenicillium* sp.	*Eurotium* sp.			
Gliocladium sp.	*Eurotium* sp.	*Eurotium* sp.	*Pseudeurotium* sp.			
Gymnoascus sp.	*Gymnoascus* sp.	*Gliocladium* sp.	*Spherodes* sp.			
Isaria farinosa	*Humicola* sp.	*Gymnoascus* sp.				
Paecilomyces sp.	*Isaria* sp.	*Melanospora* sp.				
Penicillium sp.	*Melanospora* sp.	*Poroconiochaeta* sp.				
Talaromyces sp.	*Paecilomyces* sp.	*Pseudeurotium* sp.				
Verticillium tenerum.	*Talaromyces* sp.	*Spherodes* sp.				
	Verticillium tenerum	*Talaromyces* sp.				
	Xylaria hypoxylon	*Verticillium* sp.				
		Xylaria sp.				

3.3 DISCUSSION

The isolation technique in this study was adapted from that of Furuya and Naito (1979[b]) who used 65% etanol as a selective agent for the isolation of Ascomycetes. The study of activation of germination of ascospores by Furuya and Naito in 1980, showed phenol to be the most effective activating agent. Furthermore, Greald *et al*. (2004) suggested using a 2% phenol solution as a standard method for selective recovery of ascomycetes from soil. In this study, however, a 2% phenol treatment was not sufficient to eliminate overgrowth by mucoraceous fungi and these isolates recovered were usually either sterile or anamorphic fungi. Higher concentration of phenol; 3% to 8%, (8% being the maximum solubility of phenol in water (Carey, 2003)) were consequently used in an attempt to select for ascomyceteous fungi.

In this study an increase in phenol concentration above that used by Furuya and Naito (1980) decreased the frequency of isolation of Mucorales and fungi with thin-walled propagules and increased the proportion of Ascomycetes. Ascosporic fungi often have a thick-walled ascocarp which could protect the ascospores within. Furthermore, some of the ascospores themselves are thick-walled unlike many common soil fungi which have thin-walled propagules and are unlikely to survive higher phenol concentrations (Bills and Polishook, 1993). Occasionally, common soil fungi also survived at high phenol level, probably because they are situated deep within particles and so are protected (Gerald *et al*. 2004).

Treatments with 3% and 4% phenol were found to be the most useful for the soil partial pasteurization method since these concentrations yielded a greater variety of fungal isolates and Ascomycetes were more frequently isolated. At higher phenol treatments (5% to 8%) fewer fungal isolates were retrieved and most of these were also retrieved at lower phenol levels.

Soil texture and organic matter content can affect the results of phenol sterilization. Fine-textured soils are sterilized more efficiently than those of coarse texture (Gerald *et al*. 2004).

Temperature is another major factor affecting the growth of fungi. The optimal growth temperature was found to be 20 - 25 °C (**Figures 3.1**-**3.3**) which is the optimal temperature for the growth of most fungi (Zak and Wildman, 2004). At the end of the 28 day observation period, plates from 5 and 37 °C incubation had less than 10 fungal colonies present but when allowed to sit at 25 °C for a week further colonies were observed, indicating retardation rather than death of the fungal propagules.

From the treated soil plates a total of 407 distinguishable isolates were grown and extracts subjected to bioassays as discussed in **Chapter 4**.

Chapter 3: Development of a selective isolation technique for Ascomycetes

Chapter 4

Bioactivity and chemical characterization of culture extracts

4.1 BIOACTIVITY SCREENING OF CULTURE EXTRACTS

A total of 407 isolates were cultured on MYPA for 30 days and the resultant cultures extracted with ethyl acetate (EtOAc) to yield a total of 407 fungal extracts. All 407 extracts were assayed in the quick screen against P388 cells, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. Extracts that inhibited the growth of P388 cells >80% were considered active extracts and were further assayed to determine the concentration required to inhibit the growth of P388 cells by 50% (IC_{50}).

Of the 407 extracts, 123 were active against P388 cells, 40 extracts were active against *B. subtilis* (<20% cell viability) and 19 extracts active against *C. albicans* (<20% cell viability). None of the 407 extracts tested were active against *P. aeruginosa*.

Of the total of 123 extracts that were active against P388 cell in the quick screen assay, 71% of extracts showed IC_{50} values <12,500 ng/mL. These 71% were derived from fungal isolates obtained from different phenol concentrations see **Table 4.1**.

Table 4.1: Summary of active extracts derived from fungal isolates obtained from each phenol concentration.

Phenol concentration	Number of active extracts
2%	22 (5.4%)
3%	25 (6.1%)
4%	16 (3.9%)
5%	13 (3.1%)
6%	5 (1.2%)
7%	4 (1%)
8%	2 (0.5%)
Total	**87 (71%)**

All 87 extracts had been prioritized and investigated. These extracts were then subjected to HPLC screening for separation of compounds present in the extract.

4.2 HPLC SCREENING OF CULTURE EXTRACTS

HPLC screening for significant peaks provides a basis for selection of extracts for further investigation. Detection was by both evaporative light scattering detection (ELSD) and UV (DAD), as shown in **Figure 4.1**. The HPLC-ELSD profile was used to assess the relative quantities of each of the metabolites present in each of the extracts (Higgs *et al*. 2001). The UV chromophore (**Figure 4.2**) and retention time (R_t) of the compound was used in conjunction with the in-house HPLC-UV/R_t library database to distinguish known compounds from unknown compounds. This is based on a combination of matching the UV profile **and** the retention time. If all significant peaks resulted in "known hits", further work on that extract was discontinued. This is the first step of the dereplication strategies used in this

study. Further details of dereplication strategies are discussed in **Section 4.3: Dereplication**.

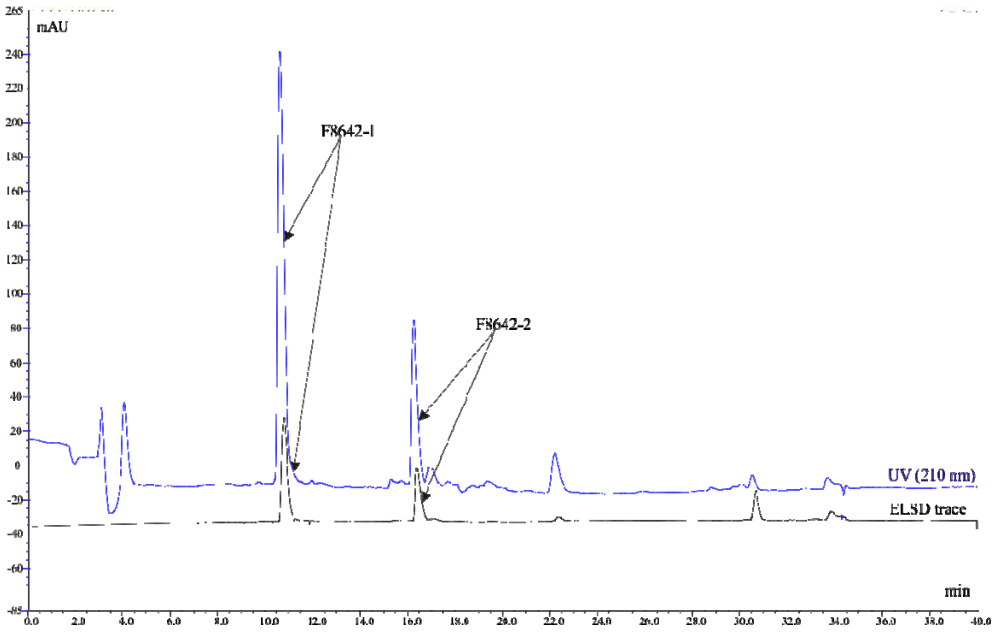


Figure 4.1: HPLC chromatogram of crude extract F8642 showing the two main peaks F8642-1 and F8642-2. UV detection 210 nm (blue) with ELSD (black) comparison.

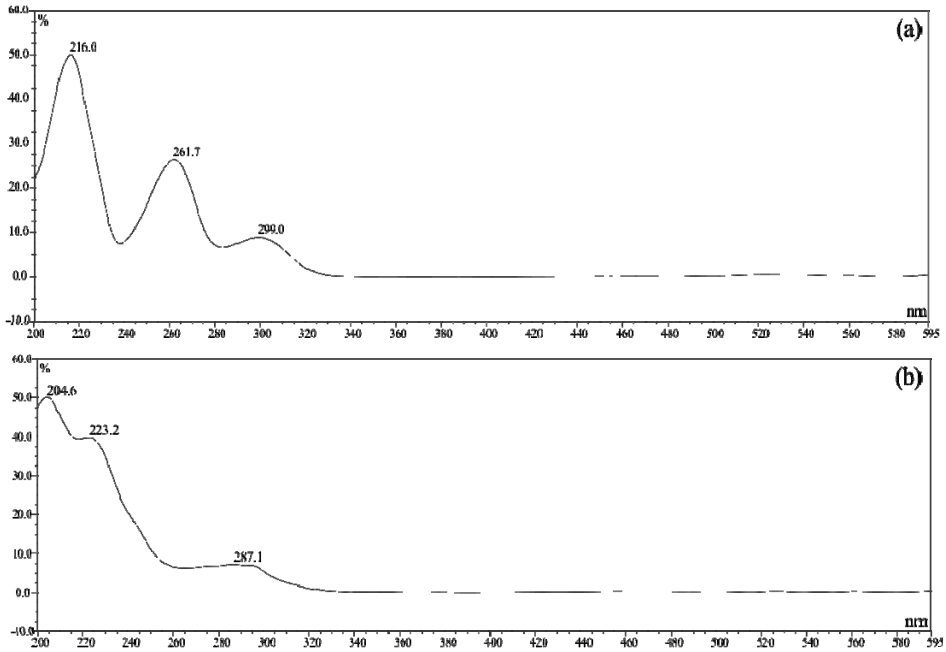


Figure 4.2: UV absorption (nm) in % of UV-max of peak F8642-1 (a) and F8642-2 (b).

4.3 DEREPLICATION

Rapid identification of natural products is an important step to distinguish known and unknown compounds and allowing the exclusion of known compounds at an early stage. The natural products chemistry group at the University of Canterbury has focused on natural products of fungal and marine origin and the search for new bioactive natural products has been aided by the development of new, effective and less time consuming methodologies for dereplication.

The group's approached to dereplication utilizes a combination of HPLC-UV and an in-house HPLC-UV/R_t library database for known fungal metabolites using the Chromeleon software on a Dionex analytical HPLC. MS analysis and capillary NMR probe (capNMR) are also utilised. These methods are all used in conjunction with the combined Marinlit/Antibase database known as AntiMarin (Lang *et al*. 2006).

4.3.1 *Dereplication using HPLC-UV library database and MS analysis*

An in-house HPLC-UV/R_t library database was created using the Chromeleon software on a Dionex analytical HPLC. Two libraries are combined in the database; an "all compound library" and a "known compound library".

Dereplication of fungal metabolites using the library databases is carried out by establishing whether any of the significant peaks in the extract shows a

match for both the UV chromophore and retention time (R_t) with any compounds present in the library. Peaks which showed a match with a known compound are then confirmed by MS analysis. Examples of dereplication using the HPLC-UV/R_t library database and MS analysis are given below.

Example 1: Extract F7342

Extract F7342 derived from a *Eurotium* sp. showed one significant bioactive peak eluting at 12.7 min in the HPLC screening (**Figure 4.3**). A search in the HPLC-UV/ R_t library database of the UV chromophores and R_t of this peak showed matches with several known compounds, with the highest match being for brefeldin A (**Figure 4.4**). The relative R_t window in the search profile was set to 13 min. The metabolite in F7342 eluted at 12.7 min and the brefeldin reference eluted at 13.1 min. Other compounds, as listed, also had close matches to the peak at 12.7 min in F7342.

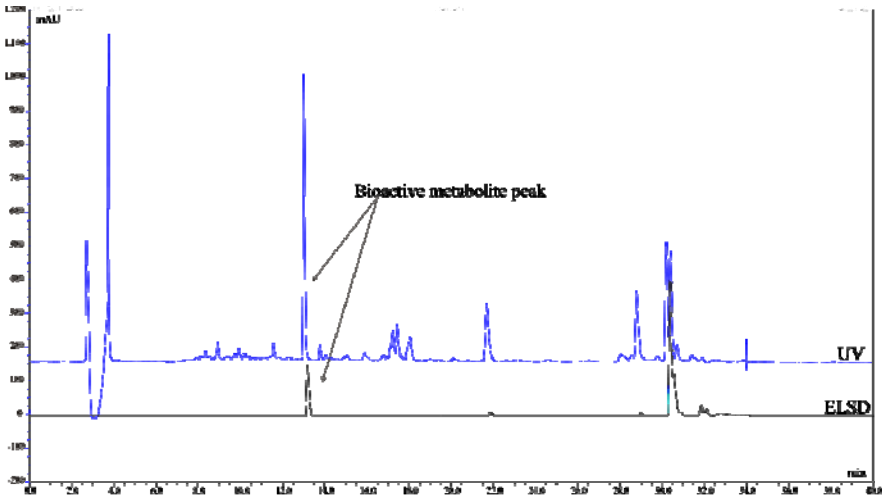


Figure 4.3: HPLC chromatogram of the crude extract F7342. UV detection (blue) with ELSD (black) comparison.

	Name	Match	Library	Ret.Time[mi	ID	#Extrema
1	Brefeldin A	999.60	Known active	13.102	A-71	0
2	bisdethiodi(methylthio)-aranotin	993.15	Known active	13.944	A-97	1
3	Waol-A	992.31	Known active	13.309	A-150	1
4	Cyclopenol	982.70	Known active	10.688	A-73	1
5	Clonostachydiol	977.49	Known active	10.826	A-141	1
6	GL1-31-2 (new roselipin)	975.53	Known active	18.591	A-149	1
7	HA 23	974.53	Known active	16.482	A-81	1
8	Homologue of Valinomycin	972.94	Known active	23.282	A-18	1
9	enniatin B	972.74	Known active	24.478	A-160	1
10	Valinomycin	971.92	Known active	21.837	A-17	1
11	Leucinostatin A	971.14	Known active	17.015	A-32	0
12	melinacidin IV	968.60	Known active	14.750	A-83	2
13	4-ketoclonostachydiol	964.59	Known active	12.479	A-143	1
14	ergosterol peroxide	964.21	Known active	23.635	A-60	0
15	chetomin	958.03	Known active	15.756	A-108	2
16	T988C	953.36	Known active	14.054	A-116	2

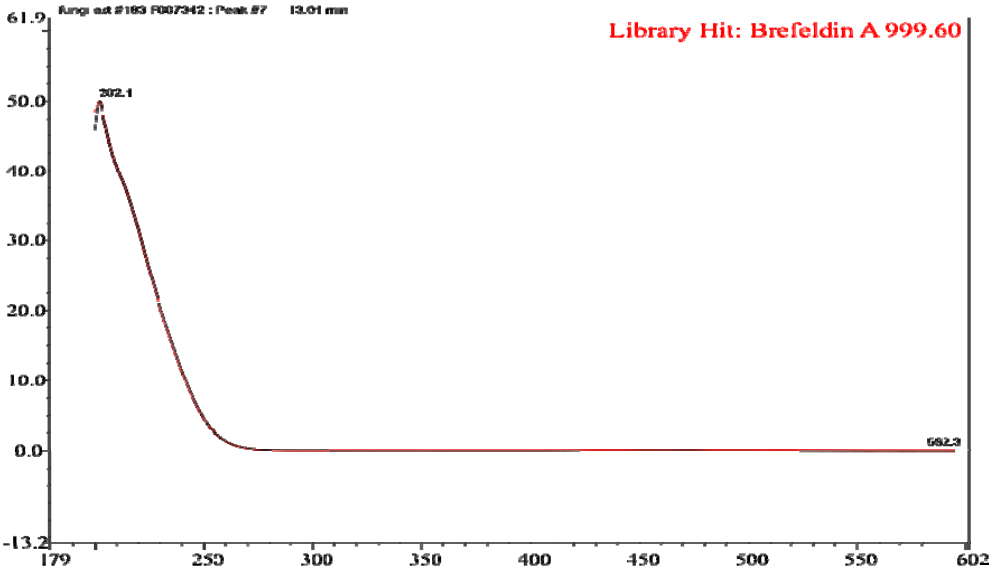


Figure 4.4: UV spectra of the peak eluted at 12.7 min (black) and brefeldin A (red) with a search in the HPLC- UV/ R_t library database.

Subsequent MS analysis of the peak at 12.7 min showed the presence of a dimeric cluster ion $[2M+H]^+$ in the spectrum that assigned the $[M+H]^+$ ion as 281.186 amu which matched with the molecular mass of brefeldin A (**Figure 4.5**) - 280.167 amu reported by Nielsen and Smedsgaard, 2003.

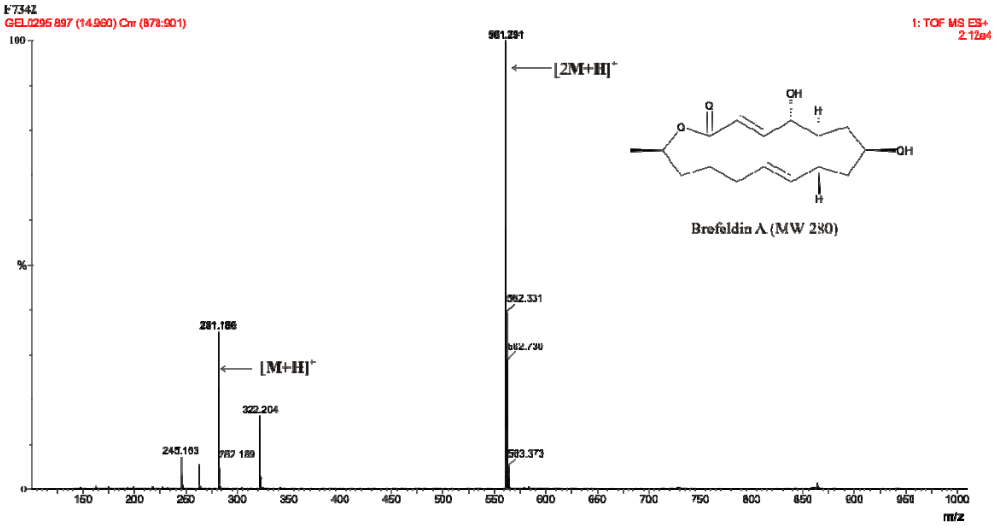


Figure 4.5: Mass spectrum of compound eluting at 12.7 min in F7342 (brefeldin A)

Since the main peak resulted in an exact match with a known compound, further work on this extract was discontinued.

Example 2: Extract F6983

Extract F6983 showed one significant peak named F6983-1 eluting at 14.39 min in the HPLC chromatogram that was subsequently shown to be active component in the bioactivity screening (**Figure 4.6**). A search of the HPLC-UV/R_t library database for the UV chromophores and R_t of this peak gave three probable matches with known compounds in the library; 2-hexylidene-3-methylsuccinic acid, Sch-642305 and 2-(2-hydroxybutyl)-pent-2-enoic acid. All three compounds shared similar UV chromophores with compound F6983-1 (**Figure 4.7**).

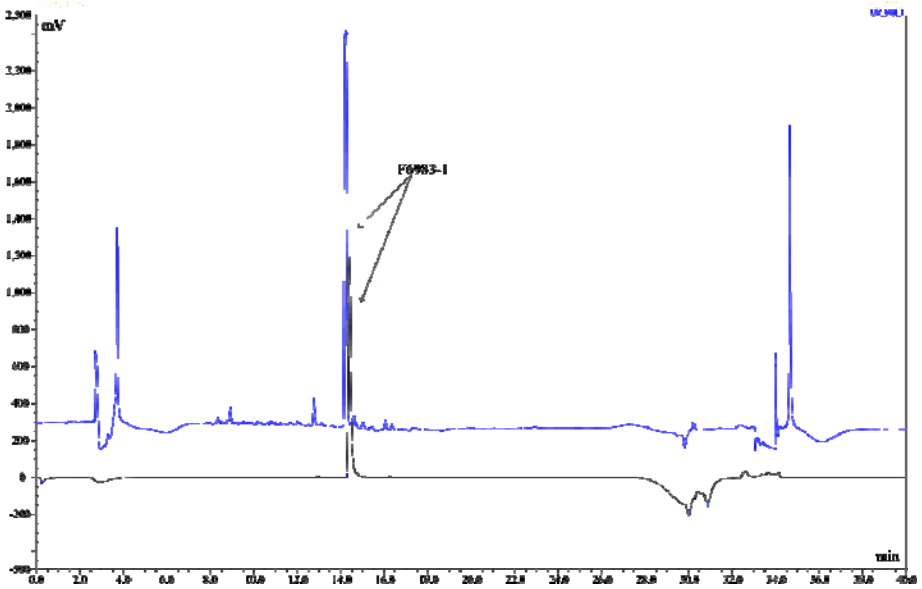


Figure 4.6: HPLC chromatogram of the crude extract F6983 showing the bioactive compound at 14.39 min. UV detection (blue) with ELSD (black) comparison.

	Name	Match	Library	Ret.Time[mi	ID	#Extrema
1	2-hexylidene-3Me-succinic acid	997.11	Known active	13.993	A-88	1
2	Sch-642305	994.07	Known active	12.799	A-162	1
3	4beat-hydroxy-8-isopropyl-5-methylene-	955.56	Known active	15.207	A-421	1
4	4-ketoclonostachydiol	946.83	Known active	12.479	A-143	1
5	Spiro-palmarumicin	942.69	Known active	14.136	A-133	2
6	cytochalasin G	928.27	Known active	14.929	A-112	3
7	2-(2-hydroxybutyl)-pent-2-enoic acid	926.62	Known active	14.547	A-51	1
8	cytochalasin X	925.29	Known active	14.357	A-113	2
9	cytochalasin y	921.78	Known active	13.089	A-79	2
10	cytochalasin Z	921.46	Known active	15.423	A-114	3
11	T988A	921.21	Known active	14.999	A-84	1
12	chaetoglobosin R	917.27	Known active	13.870	A-102	1
13	chaetoglobosin B	914.85	Known active	14.989	A-98	2
14	chaetoglobosin D	913.32	Known active	14.549	A-99	2

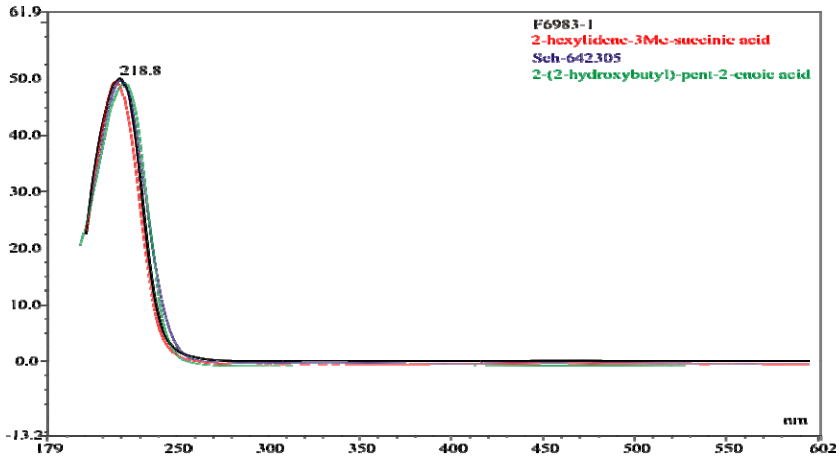


Figure 4.7: UV spectra of the peak F6983-1 (black), 2-hexylidene-3-methylsuccinic acid (red), Sch-642305 (blue) and 2-(2-hydroxybutyl)-pent-2-enoic (green) with a search in the HPLC- UV/R_t library database.

MS analysis showed that compound F6983-1 had a molecular mass $[M+H]^+$ of 215 amu which matched with the reported known compound 2-hexylidene-3-methylsuccinic acid (**Figure 4.8**) (Anderson *et al*. 1985). Hence, the main peak of this extract represented a known compound and work on this extract was also discontinued.

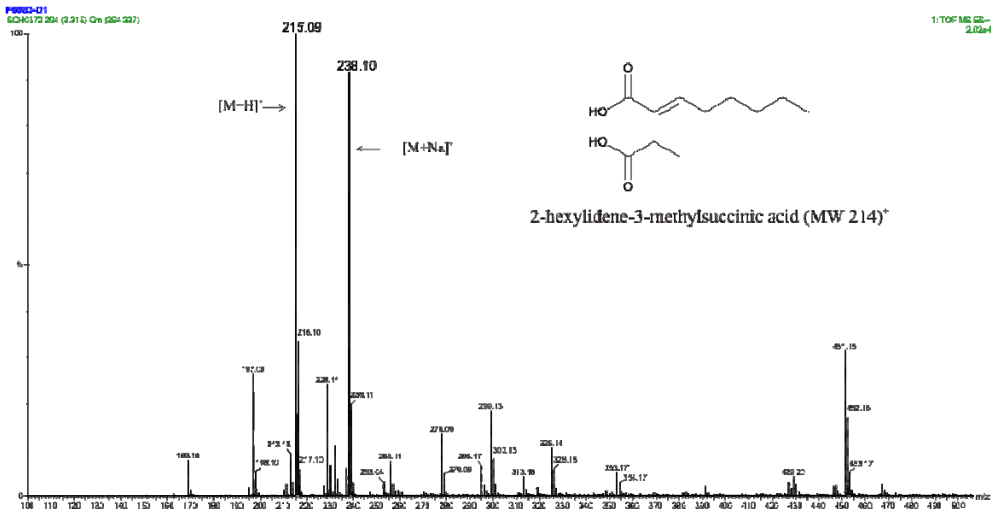


Figure 4.8: Mass spectrum of compound F6983-1 which concluded it to be 2-hexylidene-3-methylsuccinic acid.

Dereplication using the HPLC-UV library database and MS analysis is a primary method to distinguish known from unknown compounds, however, this method is not a definitive method for dereplication.

The UV spectrum only gives definitive structure information for those compounds with strong chromophores or for compounds that have absorption maxima in the range of 200-800 nm (Anderson *et al*. 2004). For compounds with commonly observed UV profiles an incorrect conclusion cn result since similar chromophores give comparable UV spectra. For example, some cytochalasin and chaetoglobosin derivatives which contain

an indole group as the backbone structure exhibit similar UV profiles (**Figure 4.9**) which correspond to the absorption maxima of indoles (216, 270 and 286 nm) (Andonovski and Stojkovlić, 2000).

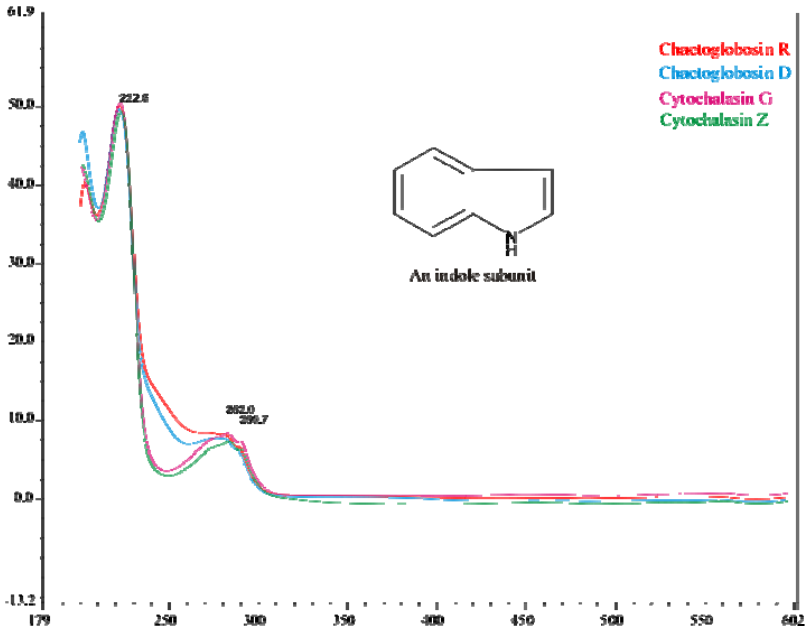


Figure 4.9: A comparison of UV spectra of cytochalasin and chaetoglobosin derivatives which correspond to an indole group in their structure.

With ESIMS analysis, the mass spectrum can contain, in addition to the molecular ion MH^+, pseudo-molecular ions such as MNa^+, MNH_4^+ or $MHCH_3CN^+$. There is also the possibility of interfering ions from minor components that ionize more readily than the component of interest. These features can lead to confusion over the actual molecular mass of the sample (Nielson and Smedsgaard, 2003; Lang *et al*. 2008). Therefore, additional data such as a ^1H NMR spectrum is necessary to deliver definitive structural information.

4.3.2 *Dereplication using the CapNMR technique*

Extracts that were not readily dereplicated using the HPLC-UV library database and MS analysis were investigated using CapNMR spectroscopy in conjunction with the AntiMarin database. With the use of a CapNMR probe, only 2-10 µg of pure compound is required for dereplication. The ^{1}H NMR spectrum can then provide specific structural information such as the number and types of a methyl group. For example, the methyl groups could be pre-set as singlets, doublets or triplets. The database also includes the ability to search on other structural features such as the type of aromatic substitution. These features are the most readily interpreted in a search in the AntiMarin database. A full account of the use of the AntiMarin database has been published (Blunt *et al.* 2007). An example of dereplication using the CapNMR technique is given below.

Example 1: Extract F8767

Extract F8767 was derived from a *Penicillium* sp. and on the basis of HPLC analysis (**Figure 4.10**) showed a presence of one major compound, denoted as F8767-1, eluting at 15.05 min. A search of the in-house UV database suggested several known compounds as possible hits with the closest match being recorded for griseofulvin and a compound F4656-9 which was griseofulvin or griseofulvin-like structure (**Figure 4.11**).

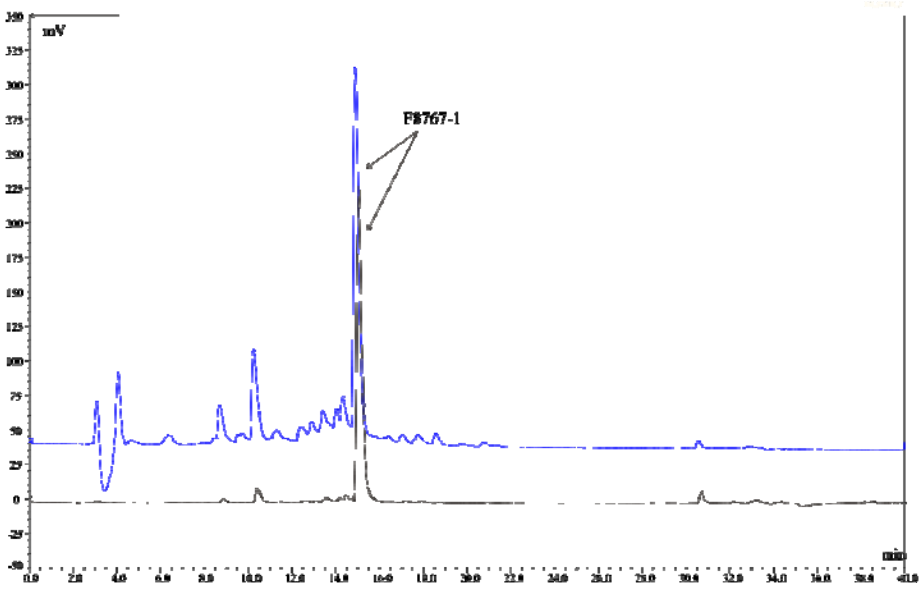


Figure 4.10: HPLC chromatogram of the crude extract F8767 showing the bioactive component at 15.05 min. UV detection (blue) with ELSD (black) comparison.

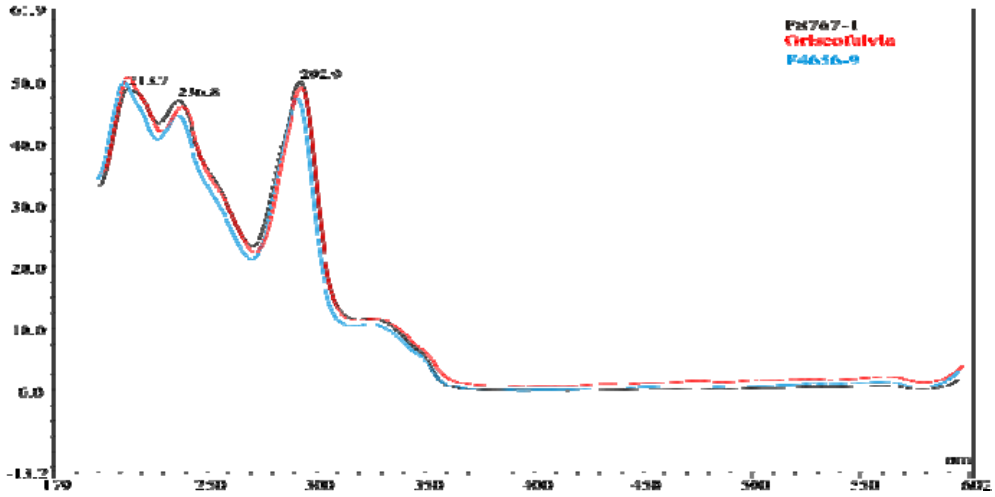


Figure 4.11: A comparison of UV spectra of compound F8767-1, Griseofulvin and F4656-9.

This compound was examined by CapNMR and the ^{1}H spectrum obtained. Compound F8767-1 displayed one doublet methyl group, three methoxy groups, one olefinic proton and one aromatic proton in the ^{1}H NMR spectrum (**Figure 4.12**).

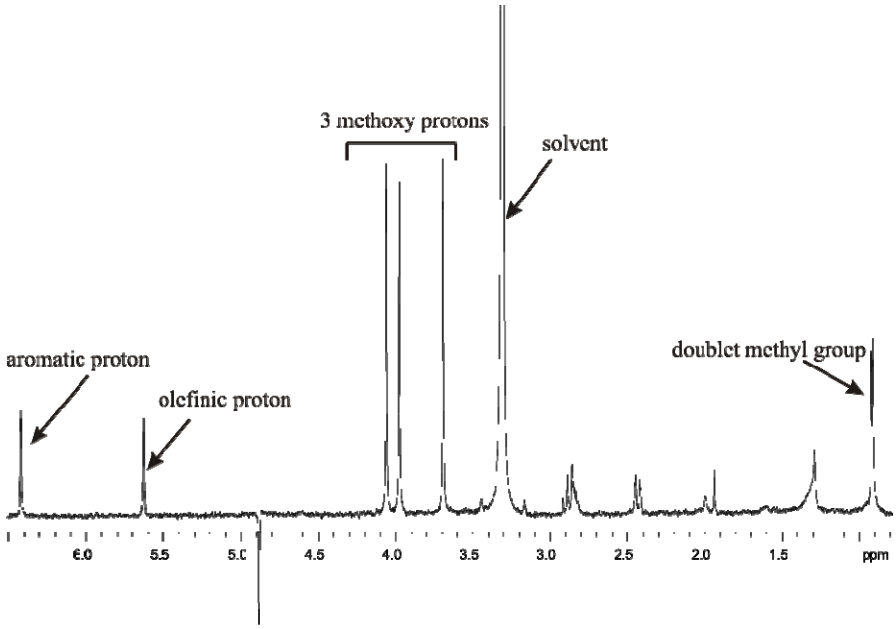


Figure 4.12: The ^{1}H NMR spectrum of 15µg of F8767-1 in 6 µL CD_3OD, recorded at 500 MHz.

These features were entered into the AntiMarin Database (**Figure 4.13**), together with the measured low resolution mass of 352 amu.

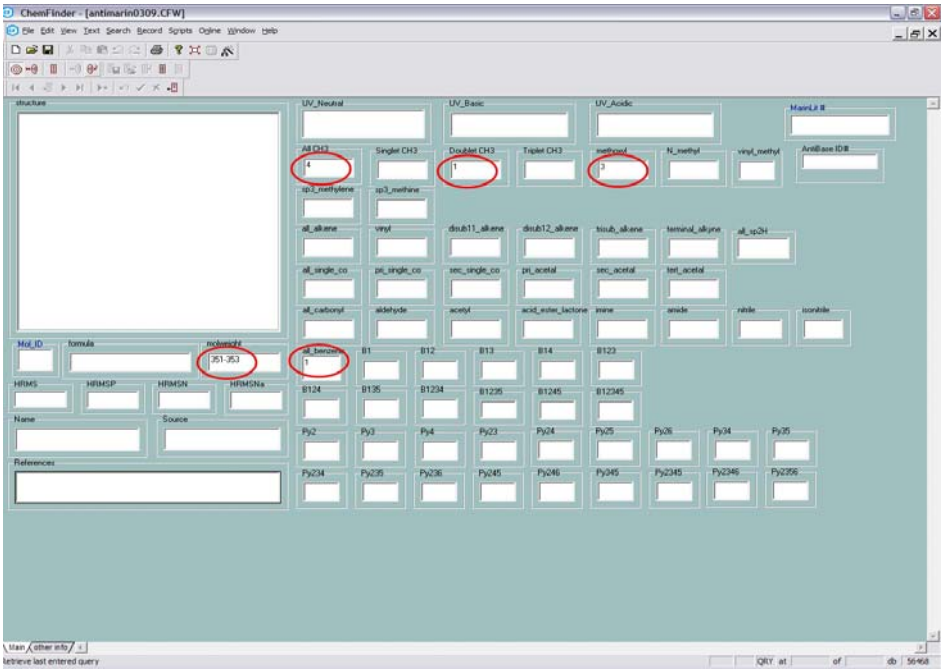


Figure 4.13: Screen shot of AntiMarin search profile for F8767-1.

Two matches were found with the same structure (**Figure 4.14**). These were both griseofulvin. Therefore, compound F8767-1 was identified as a known compound and no further work was done on this extract.

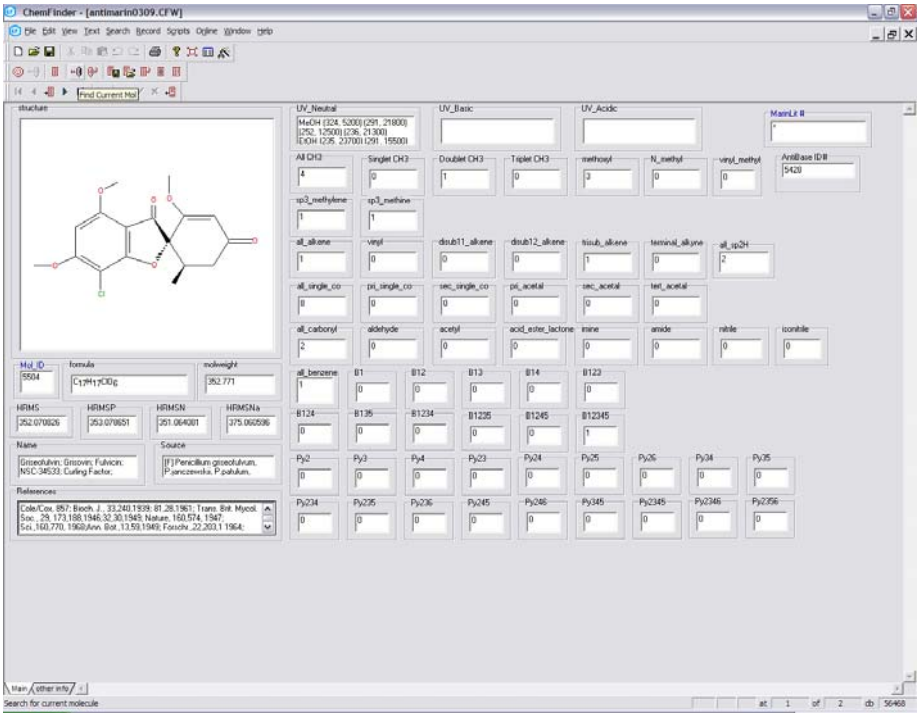


Figure 4.14: A screen shot of the AntiMarin search result for F8767-1

Using the HPLC-UV/R_t library database, MS analysis, and the CapNMR technique, 97% of the 87 extracts were eliminated from further study as all the significant bioactive peaks could be assigned as known compounds. Details of the compounds identified in the active extracts are given in **Table 4.2**. The new metabolites discovered are discussed in **Chapter 5**.

Table 4.2: Summary of dereplication of metabolites from fungal extracts.

Metabolites	Isolates	Extracts
1,3,6 trihydroxy-8-methylxanthone	702_4@30.2 (unidentified)	F7029
2-hexylidene-3-methylsuccinic acid	770_3@30.8 (*Xylaria* sp.)	F6983
	762_3@30.1 (unidentified)	F7107
	382_3@25.5 (unidentified)	F7152
	770_3@25.14 (*Xylaria* sp.)	F7167
	654_3@25.29 (unidentified)	F7236
	456_2@25.2 (*Xylaria* sp.)	F7251
	456_2@20.12 (unidentified)	F7446
4-acetyl-6,8-diOH-5-methylisocoumarin	702_3@30.10 (unidentified)	F7008
	456_3@30.5 (unidentified)	F7105
4-ketoclonostachydiol	702_3@30.1 (unidentified)	F7107
	654_4@20.2 (*Gliocladium* sp.)	F7339
	654_4@20.4 (*Gliocladium* sp.)	F7340
	654_4@20.6 (*Gliocladium* sp.)	F7342
	654_3@20.14 (unidentified)	F7403
5-methyl mellein	381_2@25.2 (unidentified)	F7169
bisvertinoid	702_5@25.9 (unidentified)	F8629
altenusin	770_3@30.7 (*Talaromyces* sp.)	F6982
andrastatin A	654_3@25.33 (*Xylaria* sp.)	F7230
beauvericin	770_2@20.4 (Beauveria sp.)	F6881
	456_2@20.11 (*Gymnoascus* sp.)	F7445
bisdethiodi(methylthio)-aranotin	654_3@25.22 (*Eurotium* sp.)	F7234
	654_4@20.12 (*Gliocladium* sp.)	F7345
bisnaphthopyrone	654_3@25.23 (*Chaetomium* sp.)	F7235
	770_2@20.13 (unidentified)	F7471
	151_5@20.9 (unidentified)	F8596
	702_7@15.4 (unidentified)	F8621
	702_5@15.5 (unidentified)	F8622
	770_5@20.8 (unidentified)	F8623
	702_5@25.13 (unidentified)	F8626
	702_5@25.15 (unidentified)	F8628
	702_5@25.9 (unidentified)	F8629
	902_3@20.7 (unidentified)	F8696

Table 4.2: (Continue)

Metabolites	Isolates	Extracts
brefeldin A	702_3@25.2 (unidentified)	F7186
	654_2@25.6 (*Penicillium* sp.)	F7210
	654_3@25.3 (*Gliocladium* sp.)	F7221
	456_3@25.4 (*Meranospora* sp.)	F7223
	NEL_3@25.7 (Telaromyces sp.)	F7226
	654_4@20.6 (*Eurotium* sp.)	F7342
	901_4@30.4 (*Eurotium* sp.)	F8678
brevianamide	770_2@30.7 (unidentified)	F7038
	770_4@25.2 (*Penicillium* sp.)	F7240
cephalochromin	381_3@25.4 (*Cephalosporium* sp.)	F7151
	702_2@25.1 (sterile)	F7164
	702_3@25.3 (*Cephalosporium* sp.)	F7171
	702_3@25.6 (sterile)	F7174
	702_3@25.11 (sterile)	F7176
	702_4@25.5 (*Cephalosporium* sp.)	F7178
	151_4@25.5 (sterile)	F7179
	702_2@20.10 (sterile)	F7319
	702_2@20.14 (sterile)	F7321
	702_2@20.15 (sterile)	F7322
	654_2@20.15 (*Gymnoascus* sp.)	F7387
	456_3@20.5 (sterile)	F7448
	456_3@20.11 (sterile)	F7452
	456_4@20.6 (sterile)	F7456
	456_4@20.9 (sterile)	F7457
	702_5@20.9 (sterile)	F8596
	702_6@20.10 (sterile)	F8598
	702_7@20.5 (sterile)	F8601
chaetoglobosin D	770_2@20.2 (*Penicillium* sp.)	F7371
	702_2@20.43 (*Chaetomium* sp.)	F7626
	701_2@20.36 (*Chaetomium* sp.)	F7680
chaetoglobosin J	770_2@20.2 (*Penicillium* sp.)	F7371
chaetoglobosin R	770_2@25.11 (unidentified)	F7212
	701_2@20.2 (*Penicillium* sp.)	F7371
	654_2@20.14 (*Penicillium* sp.)	F7386
	654_3@20.7 (unidentified)	F7400

Table 4.2: (Continue**)**

Metabolites	Isolates	Extracts
chaetoglobosin S	770_2@25.6 (*Penicillium* sp.)	F7210
	770_2@25.11 (unidentified)	F7212
	770_2@20.35 (*Chaetomium* sp.)	F8002
chetomin	654_2@30.7 (unidentified)	F7038
citreoviridin	418_2@25.7 (*Penicillium* sp.)	F7197
cladosporin	701_4@30.1 (*Cladosporium* sp.)	F6997
	702_3@30.10 (unidentified)	F7008
	456_3@30.5 (unidentified)	F7105
	654_3@25.22 (*Eurotium* sp.)	F7234
clonostachydiol	702_3@30.11 (unidentified)	F7009
curvularin	770_3@30.7 (*Talaromyces* sp.)	F6982
cycloaspeptide	702_2@30.1 (unidentified)	F7002
cycloaspeptide A	418_2@25.6 (*Aspergillus* sp.)	F7467
cytochalasin D	901_4@30.1 (unidentified)	F8646
cytochalasin G	381_2@25.1 (unidentified)	F7147
	381_2@20.6 (unidentified)	F7303
	381_2@20.2 (*Pseudeurotium* sp.)	F7564
	903_2@15.2 (unidentified)	F8713
	903_4@15.1 (unidentified)	F8714
	903_3@20.2 (unidentified)	F8724
cytochalasin X	654_2@30.9 (*Pseudeurotium* sp.)	F6908
deacetylzygosporin G	702_2@30.1 (unidentified)	F7002
dehydrosterigmatocystin	418_3@25.1 (unidentified)	F7463
	418_3@25.2 (unidentified)	F7464
desmethyl-lasiodiplodin	702_3@30.11 (unidentified)	F7009
	701_7@15.1 (unidentified)	F8619
	902_2@30.6 (unidentified)	F8642
	902_2@30.10 (unidentified)	F8657
enniatin B	901_4@37.6 (*Fusarium* sp.)	F8639
ganoderenic acid C	903_4@30.4 (unidentified)	F8676
griseofulvin	456_4@20.1 (*Xylaria* sp.)	F8591
	902_4@30.2 (*Penicillium* sp.)	F8684
	902_4@20.2 (*Aspergillus* sp.)	F8722
	903_6@20.8 (*Xylaria* sp.)	F8766
	903_6@20.9 (*Penicillium* sp.)	F8767

Table 4.2: (Continue)

Metabolites	Isolates	Extracts
gyroporin	770_4@25.1 (unidentified)	F7163
	654_2@25.6 (*Penicillium* sp.)	F7210
harzianolide A	770_3@20.4 (*Trichoderma* sp.)	F7398
hirsutide	702_2@25.1 (unidentified)	F7164
meleagrin	418_2@25.4 (*Penicillium* sp.)	F7196
melinacidin IV	654_3@25.33 (*Xylaria* sp.)	F7230
mellein	701_2@30.4 (unidentified)	F6987
N-acetyltyramine	702_4@30.2 (unidentified)	F7029
paecilosetin	770_2@20.27 (*Paecilomyces* sp.)	F7389
	903_5@30.4 (*Paecilomyces* sp.)	F8649
penicillic acid	456_2@25.14 (*Penicillium* sp.)	F7256
	418_2@25.6 (*Aspergillus* sp.)	F7467
phomosine A	770_3@25.33 (*Xylaria* sp.)	F7230
spiro-palmarumicin	770_3@25.11 (unidentified)	F7229
sterigmatocystin	701_2@30.8 (*Aspergillus* sp.)	F6990
	701_2@25.1 (unidentified)	F7180
	418_3@25.55 (unidentified)	F7213
ternatin	$654L_3$@25.29 (unidentified)	F7236
viridicatol	654_2@30.6 (*Penicillium* sp.)	F7037

4.4 DISCUSSION

The results from the quick screen assays showed that of the 407 extracts, 123 extracts were active against P388 cells, 40 extracts were active against *B. subtilis*, 19 extracts were active against *C. albicans*, and none of the extracts were active against *P. aeruginosa*. There is an implication here that those 284 extracts which displayed no activity in any assay were inactive, but this is incorrect as these extracts might possess other than these activities from the few assays that were utilized at the University of Canterbury.

The presence of secondary metabolites in extracts can be estimated by ELSD HPLC analysis. From the data in **Table 4.1**, some active metabolites, for example brefeldin A, cephalochromin and 2-hexylidene-3-methylsuccinic acid, were frequently found in different extracts derived from the same fungal type, but derived from different soil samples. Furthermore, it is not unusual for some secondary metabolites to be produced by a number of different fungal species, which may or may not be phylogenetically related (Frisvad, 1989), e.g., griseofulvin is produced by *Aspergillus* sp., *Penicillium* sp., and *Xylaria* sp. Some fungal species such as *Xylaria* sp., *Chaetomium* sp., and *Penicillium* sp. have the ability to produce a wide range of diverse compounds and they are considered good candidates for the OSMAC (one strain many compounds) approach which was adopted for further work with some fungi and will be discussed in **Chapter 5**.

Effective dereplication methods are essential in identifying compounds and eliminating known compounds rapidly (Sprogøe *et al.* 2007). The HPLC-UV/ R_t library database only gives definitive structure information for compounds containing strong and unique chromophores. In addition, compounds which contain the same sub-structural unit may give similar UV spectra (Carey, 2003) and can lead to confusion in identifying compounds. In this study, for example, the UV spectra of chaetoglobosin and cytochalasin derivatives gave similar UV profiles due to an indole subunit (**Figure 4.9**). A conclusive molecular weight cannot always be obtained from a single positive ESIMS experiment. Too often pseudo-molecular ions such as MH^+, MNa^+, MNH_4^+ or $MHCH_3CN^+$, or the presence of interfering

ions from minor components which ionize more readily than the component of interest can lead to confusion over the actual molecular weight of the bioactive compound (Lang *et al.* 2008). This phenomenon was seen for compound F6983-1 (**Figure 4.8**) which was identified as 2-hexylidene-3-methylsuccinic acid. Therefore, the dereplication approach using HPLC-UV/R_t and MS analysis works efficiently if compounds have a characteristic UV spectrum, simple mass and not too many hits in the AntiMarin database.

The limited amount of materials from extracts is another issue in natural products studies. With the CapNMR technique only 5-10 μg of pure compounds are required to obtain a ^{1}H NMR spectrum (Gronquist *et al.* 2005; and Mitova *et al.* 2008[b]). The data from the ^{1}H NMR spectrum and mass spectrometry provided useful information for subsequent AntiMarin database searching to determine the identity of the bioactive compounds of interested.

The dereplication method develop by the Marine Group not only saves on the time taken to determine whether the compounds is known or unknown, but it also markedly reduces the amount of extract required. More dereplication examples using the CapNMR/database method are described in the following chapter, together with examples where the identification of new bioactive compounds was achieved.

Chapter 5

Effect of culture conditions and elicitors on metabolite production

5.1 INTRODUCTION

For decades, natural product researchers have been developing strategies to discover additional novel bioactive compounds either from animals, plants or microorganisms. The first option is to access the biodiversity and explore untapped natural resources for discovering novel bioactive compounds. Another option, especially for microorganisms, is to elevate metabolite production by influencing the biosynthetic pathways for secondary metabolites of such organisms. A systematic alteration of cultivation parameters; such as media composition, pH value, temperature, addition of enzyme inhibitors and oxygen supply, is called an OSMAC (one strain many compounds) approach (Bode *et al.*, 2002). This approach represents a powerful tool that has potential to yield a variety of metabolites from different microbes; bacteria, actinomycetes and fungi either from soil or marine origin (Bode *et al.*, 2000[a]; Christian *et al.*, 2005; Groud *et al.*, 2002; Puder *et al.*, 2001).

Based on the HPLC profiles of the 87 active extracts described in **Chapter 4**, seven fungal isolates were selected for the OSMAC approach. Apart from bioactivity and HPLC traces, further criteria were considered for selection of these seven fungal isolates. From previous reports of the secondary metabolites produced by *Chaetomium globosum*, *Penicillium* sp., *Pseudeurotium zonatum* and *Xylaria* sp. (Ariyo *et al.*, 1997; Whalley and Edwards, 1999; Paranagama *et al.*, 2007) these fungi are classified as productive genera and hence deemed to have the potential to produce a greater variety of metabolites. The rare and unstudied isolates of *Chaetomium trigonosporum* and *Bombardia* sp. were also selected. Furthermore, an unidentified white isolate, which was recovered from a high phenol treated soil, was also selected. Selected isolates are shown in **Figures 5.1 (a)** and **(b)**.

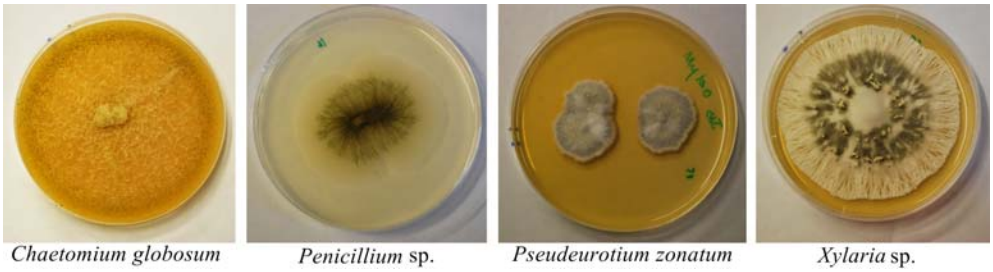


Figure 5.1 a) Examples of isolates selected for the OSMAC approach based on productive genera

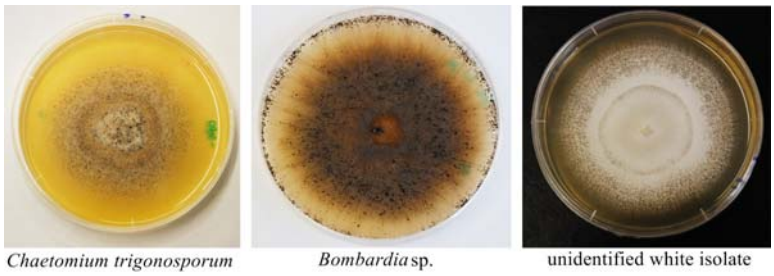


Figure 5.1 b) Examples of isolates selected for the OSMAC approach based on resistance to phenol and rarity.

5.2 EFFECT OF CULTURE CONDITIONS ON METABOLITE PRODUCTION

Three out of the seven fungal isolates; *P. zonatum*, *C. globosum* and *C. trigonosporum*, were chosen as representatives for a study of the effects of culture conditions on metabolite production. The main aim of this study was to observe the effects of different culture parameters, such as media composition, period of fermentation, state of fermentation (solid or liquid phase fermentation) and temperature, on metabolite production and to determine optimal conditions for growth and metabolite production. Also the aim was to establish a set of standard culture conditions for the selected seven fungi for a later study on the effect of elicitors on metabolite production (**Section 5.3)**. Results of the effect of these culture conditions on the growth of these three isolates is given in **Appendix III**.

5.2.1 Effect of state of fermentation on production of cytotoxic metabolites

Two media bases were used: - a nutrient rich medium (Malt Yeast Peptone (MYP)) and a nutrient deficient medium (¼ strength Sabouraud Dextrose (SD)). These were prepared in solid phase; Malt Yeast Peptone Agar (MYPA) and ¼ strength Sabouraud Dextrose Agar (SDA), and in liquid phase; Malt Yeast Peptone Broth (MYPB) and ¼ strength Sabouraud Dextrose Broth (SDB) (see **Section 2.4.3** in **Chapter 2** for methods). The effect of solid and liquid phase fermentation on the production of cytotoxic compounds was ascertained by P388 assay and the results are given in **Table 5.1**.

Table 5.1: P388 activity of extracts from *P. zonatum*, *C. globosum* and *C. trigonosporum* cultured under different conditions

Fungi	Cultural period (days)	Temp (°C)	Conditions	P388 IC_{50} (ng/mL) on different Media	
				MYP	SD
Pseudeurotium zonatum	20	20	Agar (85 mm Petri dish)	3,754	5,542
			Broth, static	5,711	>12,500
			Broth, shaken	9,793	>12,500
			Broth, ½ shaken ½ static	8,431	7,916
		25	Agar (85 mm Petri dish)	4,916	2,954
			Broth, static	>12,500	4,361
			Broth, shaken	>12,500	11,376
			Broth, ½ shaken ½ static	>12,500	>12,500
	30	20	Agar (85 mm Petri dish)	1,239	1,722
			Broth, static	>12,500	11,376
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	7,494	>12,500
		25	Agar (85 mm Petri dish)	3,986	2,324
			Broth, static	9,330	10,782
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	>12,500	>12,500
Chaetomium globosum	20	20	Agar (85 mm Petri dish)	2,479	3,986
			Broth, static	10,258	11,376
			Broth, shaken	> 12, 500	> 12, 500
			Broth, ½ shaken ½ static	> 12, 500	> 12, 500
		25	Agar (85 mm Petri dish)	8,431	2,954
			Broth, static	> 12, 500	> 12, 500
			Broth, shaken	> 12, 500	> 12, 500
			Broth, ½ shaken ½ static	> 12, 500	12,253
	30	20	Agar (85 mm Petri dish)	1,975	3,431
			Broth, static	> 12, 500	> 12, 500
			Broth, shaken	> 12, 500	> 12, 500
			Broth, ½ shaken ½ static	> 12, 500	> 12, 500
		25	Agar (85 mm Petri dish)	12,253	1,035
			Broth, static	> 12, 500	> 12, 500
			Broth, shaken	> 12, 500	> 12, 500
			Broth, ½ shaken ½ static	> 12, 500	> 12, 500

* P388 IC_{50} activities ≥12,500 ng/mL are considered to be inactive.

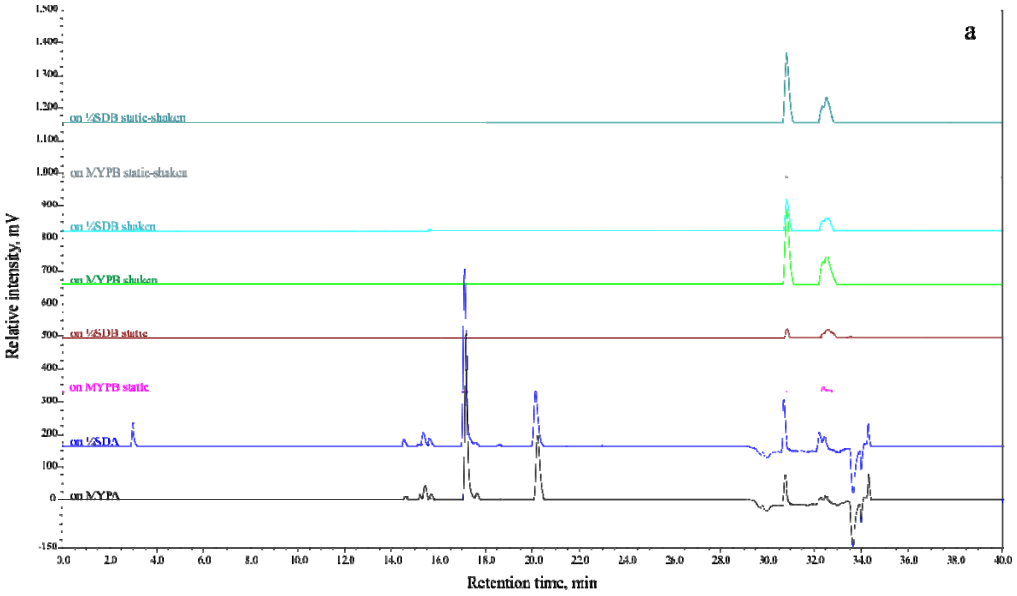
Table 5.1: (Continued)

Fungi	Cultural period (days)	Temp (°C)	Conditions	P388 IC_{50} activities (ng/mL) on different Media	
				MYP	SD
Chaetomium trigonosporum	20	20	Agar (85 mm Petri dish)	>12,500	8,951
			Broth, static	>12,500	>12,500
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	>12,500	>12,500
		25	Agar (85 mm Petri dish)	10,398	9,224
			Broth, static	>12,500	>12,500
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	>12,500	4,361
	30	20	Agar (85 mm Petri dish)	>12,500	5,220
			Broth, static	>12,500	>12,500
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	>12,500	>12,500
		25	Agar (85 mm Petri dish)	>12,500	>12,500
			Broth, static	>12,500	>12,500
			Broth, shaken	>12,500	>12,500
			Broth, ½ shaken ½ static	>12,500	>12,500

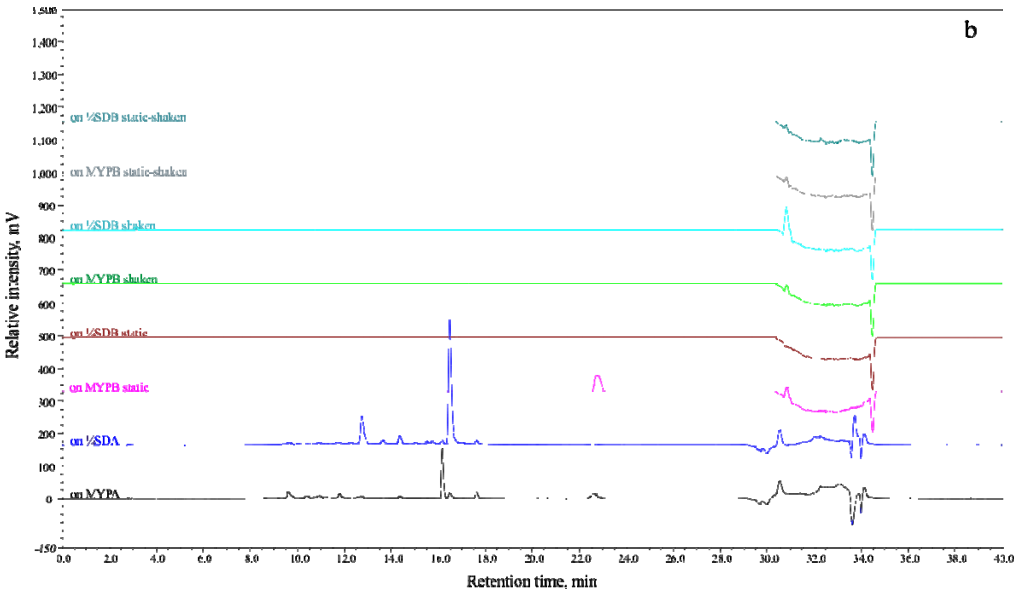
* P388 IC_{50} activities ≥12,500 ng/mL are considered as inactive.

Analysis of the results showed that solid phase fermentation favoured metabolite production in *P. zonatum*, *C. globosum* and C. *trigonosporum*. Agitation, or the combination of static and shaken conditions did not improve bioactivity. Nevertheless, the P388 IC_{50} results of *P. zonatum* fluctuated and no firm conclusions could be drawn.

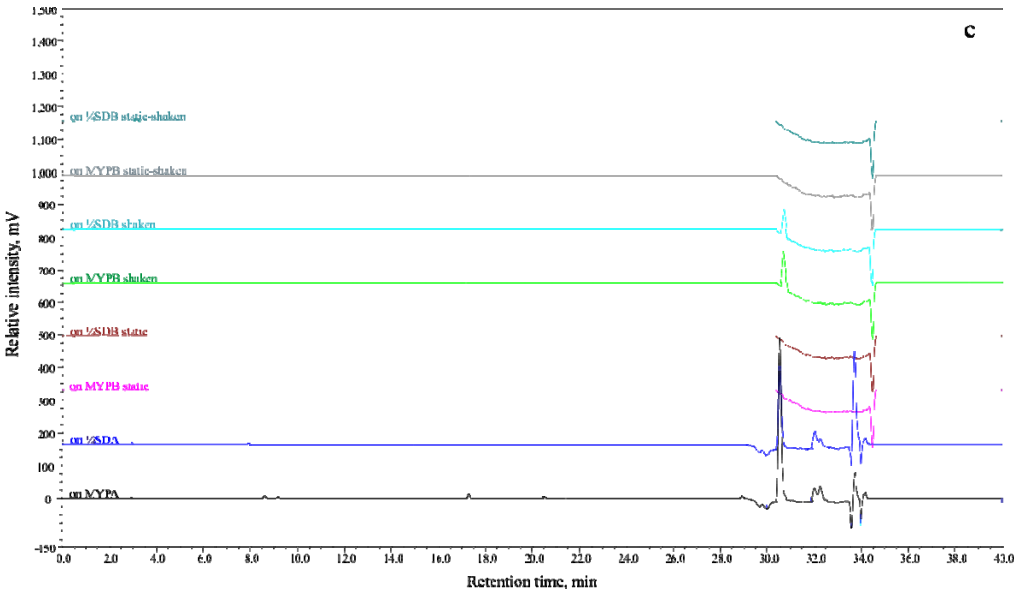
The presence of significant amounts of metabolites in the fungal extracts was detected by HPLC screening. HPLC traces showed no metabolite peaks from liquid media culture (**Figure 5.2a-c**).



(a): HPLC traces of *P. zonatum* grown at 20 °C for 20 days on differing media and conditions**.**



b): HPLC traces of *C. globosum* grown at 20 °C for 20 days on differing media and conditions**.**



c): HPLC traces of *C. trigonosporum* grown at 20 °C for 20 days on differing media and conditions**.**

Figure 5.2: HPLC traces of extracts from *P. zonatum*, *C. globosum* and C. *trigonosporum*.

5.2.2 Effect of temperature and cultivation period on bioactive metabolite production

The three selected fungi were cultured on solid media (MYPA and SDA) at four different temperatures; 10, 20, 25 and 30 °C. Cytotoxic and antimicrobial metabolite production were assessed at 10, 20, 30 and 40 days. Results are shown in **Table 5.2**. The effect of temperature and cultivation period on growth was also noted results are given in **Appendix III**.

Table 5.2: Bioactivities of *P. zonatum*, *C. globosum* and *C. trigonosporum* under different test conditions.

Fungi	Cultural period (days)	Temp (°C)	Bioactivity of extracts on different media					
			P388 IC_{50} activities (ng/mL)		*B. subtilis (% of cell growth)*		*C. albicans (% of cell growth)*	
			MYPA	SDA	MYPA	SDA	MYPA	SDA
Pseudeurotium zonatum	10	10	14,458	9,540	>50.0	>50.0	>50.0	>50.0
		20	12,825	3,868	>50.0	>50.0	>50.0	>50.0
		25	>12,500	7,940	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	20	10	4,916	6,063	27.4	38.5	45.8	47.6
		20	3,754	5,542	34.7	31.1	21.8	37.9
		25	4,916	7,760	43.2	48.6	50.3	51.3
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	30	10	2,324	6,634	44.5	51.9	38.1	48.2
		20	2,324	1,722	27.4	31.5	21.8	23.2
		25	3,986	2,324	49.8	42.8	29.7	48.5
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	40	10	2,395	3,536	>50.0	>50.0	38.1	48.2
		20	1,574	2,867	31.4	>50.0	>50.0	23.2
		25	4,771	4,771	>50.0	>50.0	29.7	48.5
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
Chaetomium globosum	10	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	>12,500	3,986	>50.0	>50.0	>50.0	>50.0
		25	8,431	2,954	>50.0	>50.0	>50.0	>50.0
		30	>12,500	8,182	>50.0	>50.0	>50.0	>50.0
	20	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	1,775	1,167	>50.0	>50.0	>50.0	>50.0
		25	3,643	1,775	>50.0	>50.0	>50.0	>50.0
		30	3,754	1,276	>50.0	>50.0	>50.0	>50.0
	30	10	8,687	3,431	>50.0	>50.0	>50.0	>50.0
		20	>12,500	3,431	>50.0	>50.0	>50.0	>50.0
		25	>12,500	1,035	>50.0	-17.1	>50.0	>50.0
		30	>12,500	4,107	>50.0	>50.0	>50.0	>50.0
	40	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		25	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0

* P388 IC_{50} activity >12,500 ng/mL and antimicrobial activity over 50% of cell growth were considered to be inactive.

Table 5.2: (Continued)

Fungi	Cultural period (days)	Temp (°C)	Activities on different media					
			P388 IC_{50} activities (ng/mL)		*B. subtilis* (% of cell growth)		*C. albicans* (% of cell growth)	
			MYPA	SDA	MYPA	SDA	MYPA	SDA
Chaetomium trigonosporum	10	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		25	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	20	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	11,600	>12,500	>50.0	>50.0	>50.0	>50.0
		25	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	30	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	>12,500	12,764	>50.0	>50.0	>50.0	>50.0
		25	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
	40	10	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		20	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		25	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0
		30	>12,500	>12,500	>50.0	>50.0	>50.0	>50.0

* P388 IC_{50} activity over 12,500 ng/mL and antimicrobial activity over 50% of cell growth were considered to be inactive.

From the data in **Table 5.2** it was concluded that the production of bioactive compounds from *P. zonatum* and *C. globosum* were found to be best at 20 °C on both of the media used. In contrast, extracts from *C. trigonosporum* showed no bioactivity except when the fungus was grown at 20 °C on MYPA with a 20 day cultivation period. HPLC profiles of the extracts of each fungus assessed at each cultivation period at 20 °C were compared and are shown in **Figures 5.3 – 5.5**.

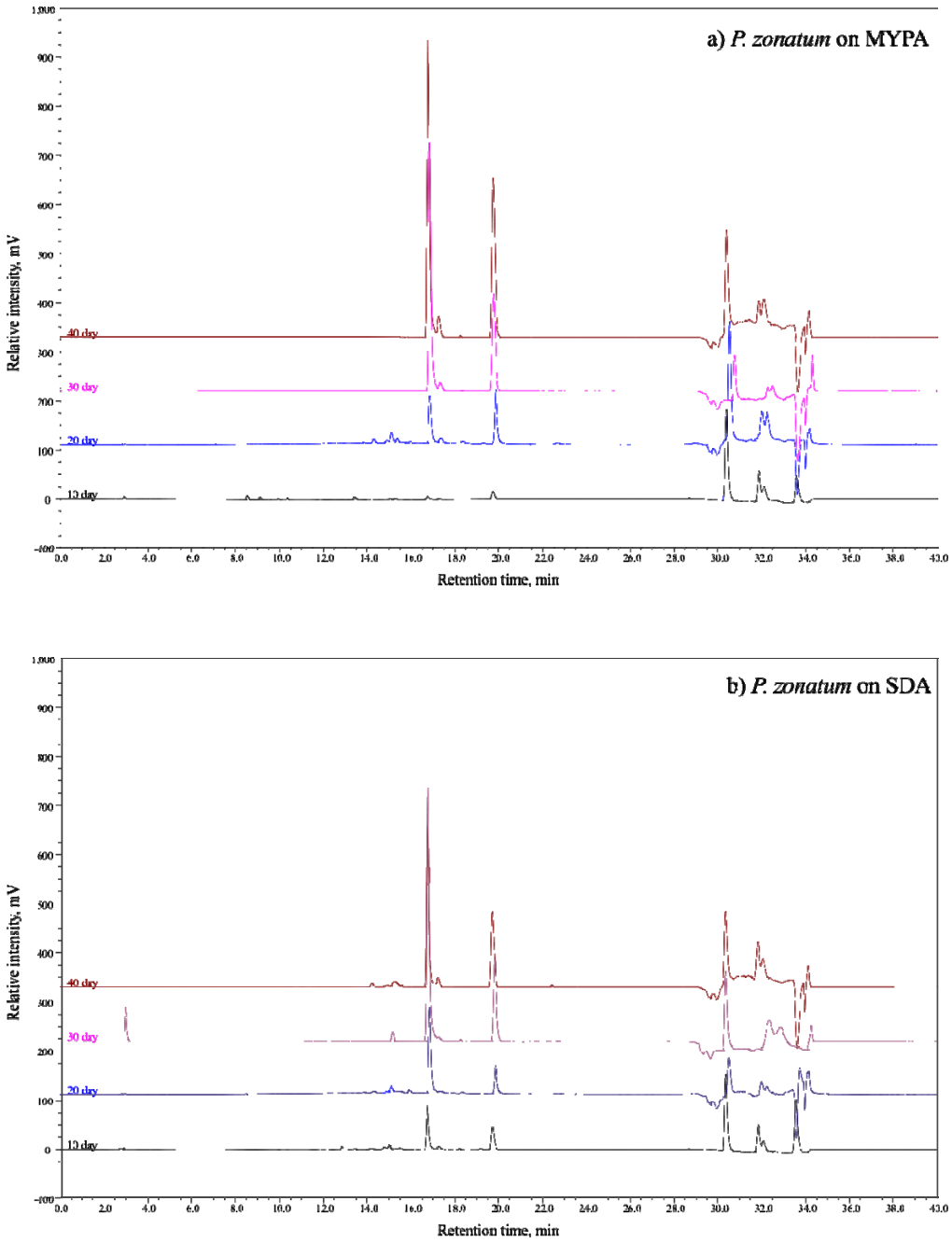


Figure 5.3: HPLC profiles of extracts from *P. zonatum* grown at 20 °C on a) MYPA medium and b) SDA medium, accessed at 10, 20, 30 and 40 days of cultivation period.

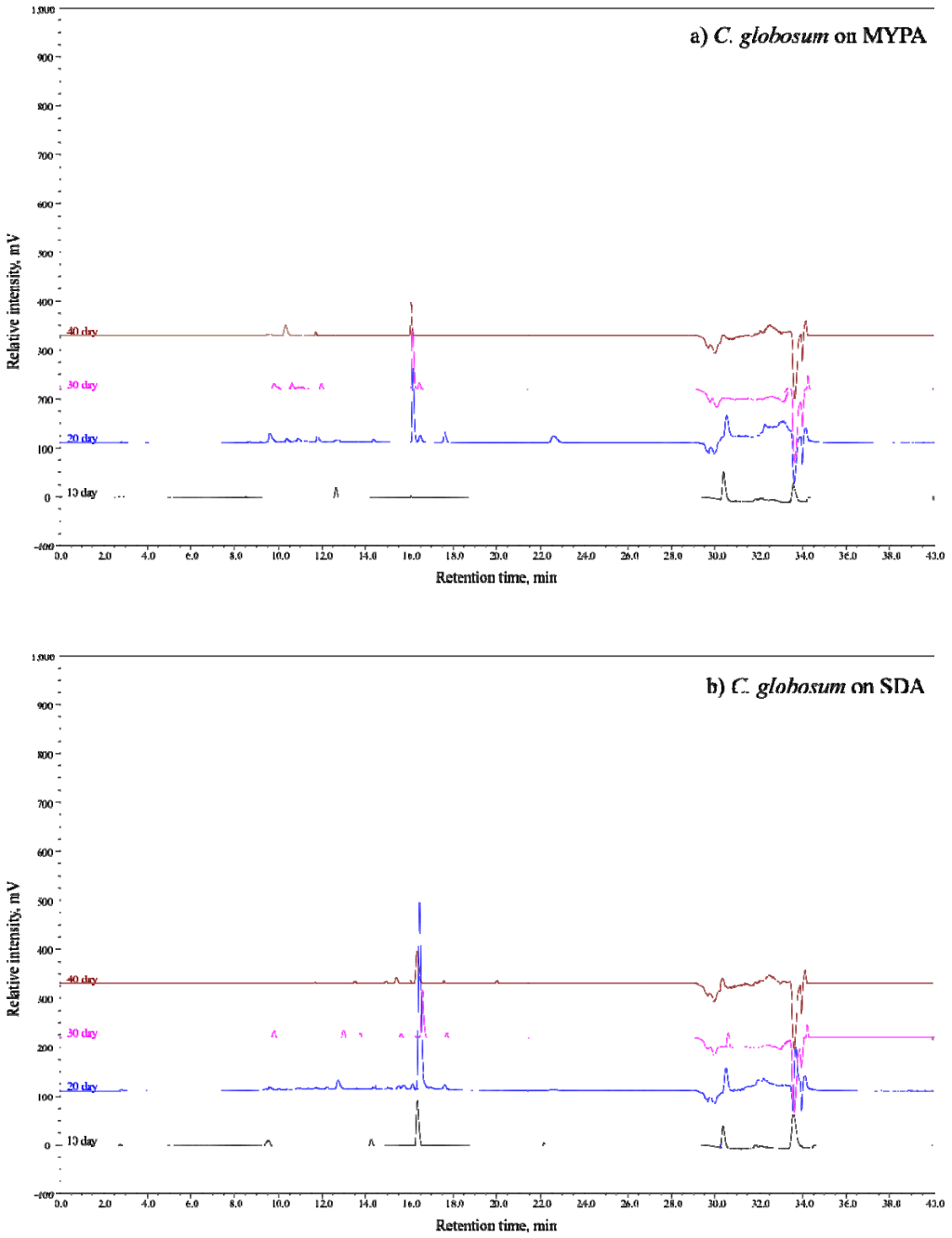


Figure 5.4: HPLC profiles of extracts from *C. globosum* grown at 20 °C on a) MYPA medium and b) SDA medium, accessed at 10, 20, 30 and 40 days of cultivation period.

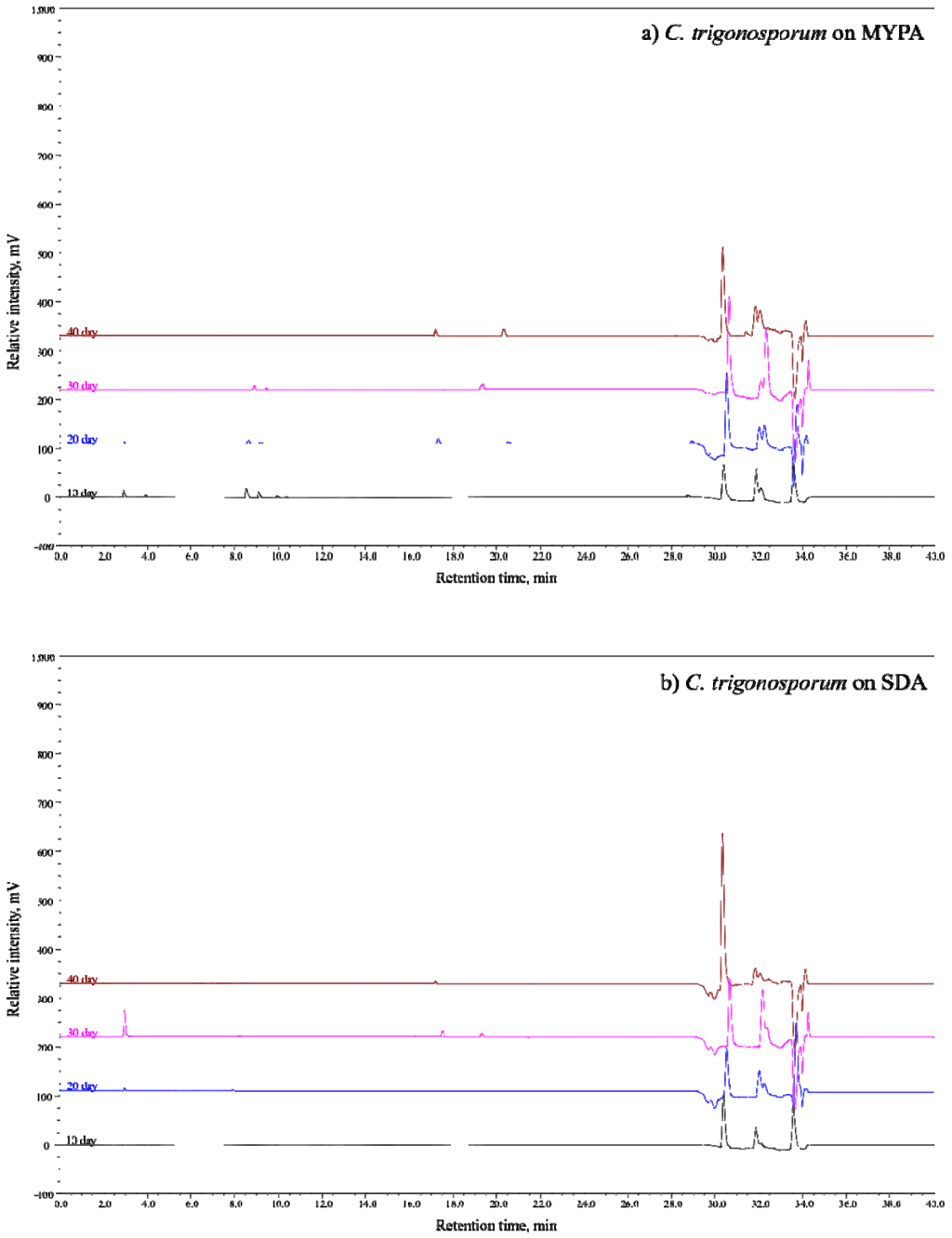


Figure 5.5: HPLC profiles of extracts from *C. trigonosporum* grown at 20 °C on a) MYPA medium and b) SDA medium, accessed at 10, 20, 30 and 40 days of cultivation period.

The HPLC profiles of *P. zonatum* showed no difference in metabolite production between the two media. A significant amount of metabolite production was first detected when the fungus was grown for 20 days on

MYPA medium and for 10 days on SDA. HPLC profiles and the bioactivity results for *P. zonatum* are shown in **Table 5.2**. A longer cultivation period increased metabolite production with a consequent increase in bioactivity (lower IC_{50} values). Details of the elucidation of compounds present are given in **Section 5.6**.

HPLC profiles of *C. globosum* (**Figure 5.4a** and **b**) showed that different metabolites were produced when the fungus was grown on different media (Details of elucidation of compounds are given in **Section 5.9**). Metabolite production was first detected at 20 days when the fungus was grown on MYPA medium, and at 10 days on SDA medium.

No significant amount of compounds was detected from the HPLC profiles of *C. trigonosporum*. Nevertheless, studies on the effects of elicitors on metabolite production are continuing for this fungus.

Metabolite production was found to be best at 20 °C and a 20 day cultivation period on both media. This in combination appeared to be an appropriate time/temperature selection for further studies on the effect of elicitors on the growth and metabolite production of the seven selected fungi.

5.3 EFFECT OF ELICITORS ON METABOLITE PRODUCTION

Recent studies have shown that minor variations in the environment or nutrition have the potential to impact the quantity and diversity of fermentation products of microorganisms (Bills *et al*. 2008). Furthermore, the use of elicitors such as antibiotics and cytoskeleton inhibitors at subinhibitory concentration has been shown to alter secondary metabolite production in actinomycetes, bacteria and fungi (Goh *et al.* 2002; Christian *et al*. 2005; Mitova *et al*. 2008[a]). The use of subinhibitory concentrations of these elicitors is based on the concept of hormesis which was defined as low-dose stimulation and high-dose inhibition (Calabrese *et al*. 2002). This is an adaptive response of organisms to low levels of stress or damage. Many studies have shown that a modulation in cell transcription occurred in the presence of antibiotics or inhibitors at low concentration, as they act as signaling rather than inhibitory agents (Hoffman *et al*. 2005; Linares *et al*. 2006; Yim *et al*. 2006).

For the next step in the current study, the seven chosen fungi were challenged with elicitors such as antibiotics and actin inhibitors to determine if secondary metabolite production was affected by subinhibitory concentration of elicitors. Elicitors used in this study are summarized in **Table 5.3**, while media preparation is given in **Appendix I**.

Table 5.3: List of elicitors used in OSMAC approach

Name	Amount presence in 1 mL agar	Mode of action
Cycloheximide (CLY)	10 mM	Interference with the translocation step in protein synthesis thus blocking translational elongation.
	5 mM	
	2.5 mM	
Histone Deacetylase Inhibitor II (HDACI II)	0.2 µM	Induces apoptosis (Glick *et al*. 1999; Marks *et al*. 2000)
	0.4 µM	
	0.8 µM	
Histone Deacetylase Inhibitor IV (HDACI IV)	0.05 µM	Reverse gene silencing (Herman *et al*. 2006)
	0.5 µM	
	1 µM	
Jasplakinolide (JAS)	0.01 µM	F-actin stabilizer (Bubb *et al*. 2000; Fenteany and Zhu, 2003)
	0.05 µM	
Latrunculin B (LB)	0.25 µg	F-actin destabilizer (Fenteany and Zhu, 2003)
	0.5 µg	
Mycophenolic acid (MPA)	1.5 µg	Enzyme inhibitor (Sweeney *et al*. 1972)
Nystatin (NYS)	1 U	Interruption of fungal cell membranes, that leads to K^+ leakage and fungal cell death
	10 U	
	100 U	
Phalloidin (PHA)	0.1 U	F-actin stabilizer (Fenteany and Zhu, 2003)
Tetracycline (TTC)	1.0 µg	Protein synthesis inhibitor
Tricyclazole (TCZ)	1.5 µg	DHN biosynthesis inhibitor

Part A: *Pseudeurotium zonatum*

5.4 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 381_4@20.2 was isolated from a 4% phenol treated soil sample that originated from Arthur's Pass (stream edge), Canterbury. The fungal strain was identified as belonging to the family Pseudeurotiaceae. Colonies were slow growing, reaching 12-16 mm in diameter after 10 days at 20 °C on malt-yeast-peptone agar (MYPA) (**Figure 5.6a**). The young colony had white aerial and submerged mycelium. Colonies became grey or dark brown when cleistothecial ascomata developing (**Figure 5.6b**); ascomata matured within 20-25 days. Ascomata globose, 70-130 µm in diameter, ostiole and ascomatal hairs were absent. Cleistothecial surface was composed of cephalothecoid plates (**Figure 5.6c**). Asci evanescent; ascospores globose, 2.0-4.0 µm in diameter (**Figure 5.6d-e**).

After comparing the characteristic of this fungal strain with the description provided by Malloch and Cain (1970), Domsch *et al*. (1980) and Sogonov *et al*. (2005), fungal strain 381_4@20.2 was identified as *Pseudeurotium zonatum*.

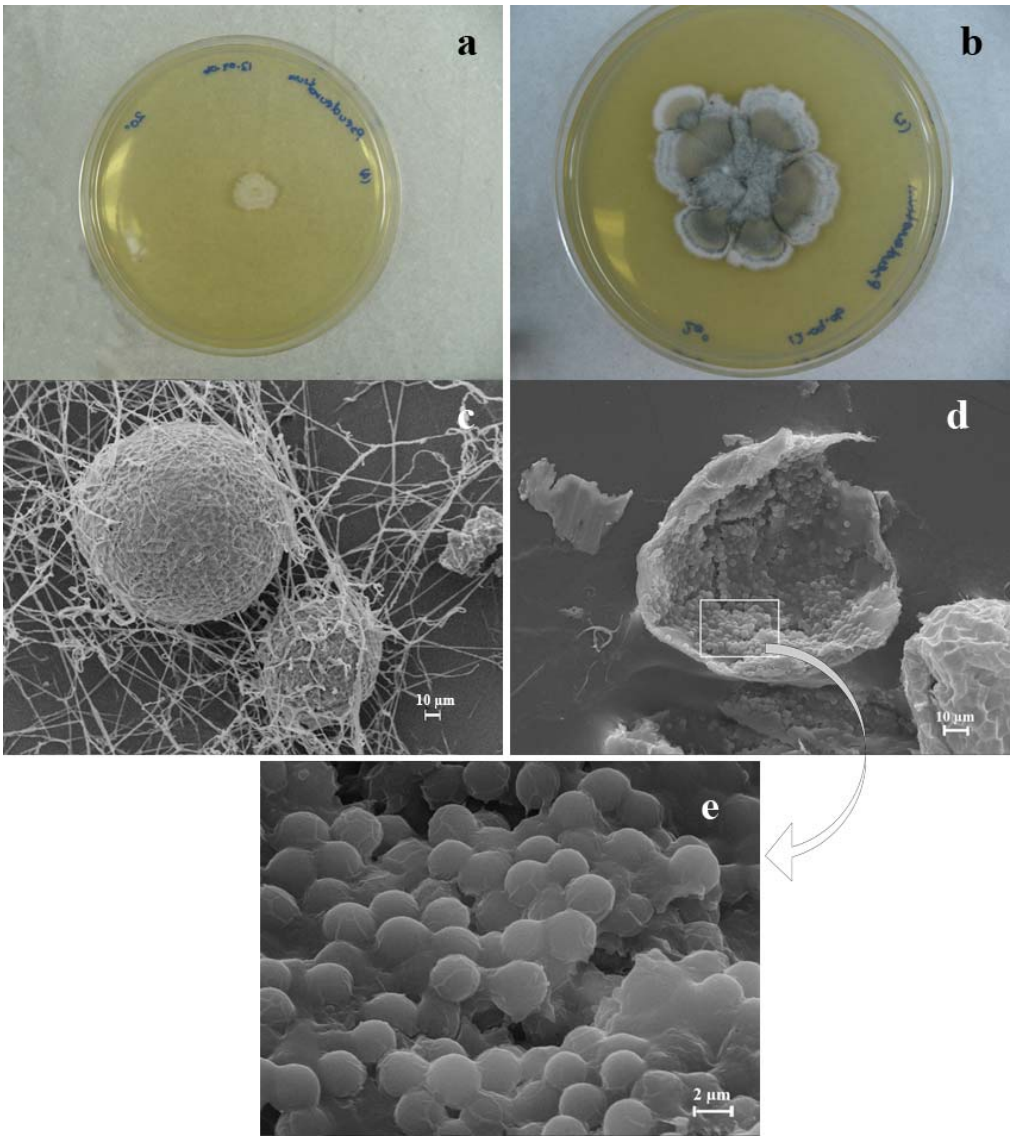


Figure 5.6: *Pseudeurotium zonatum*. **a)** 10 days culture at 20 °C on MYPA plate; **b)** 30 days culture at 20 °C on MYPA plate; **c)** SEM of cleistothecium (bar = 10 µm); **d)** SEM of fractured cleistothecium and ascospores (bar = 10 µm); **e)** ascospores (bar = 2 µm).

5.5 EFFECT OF ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.5.1 Effect of elicitors on the growth of *P. zonatum*

P. zonatum was grown on MYPA and SDA media at 20 ° C for 20 days. The colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of mycelial growth of *P. zonatum* in the presence of elicitors in MYPA and SDA media is shown in **Figures 5.7** and **5.8** respectively. Ascocarp production was observed and presented in **Table 5.4**

Table5.4: Observation of ascomata production at 20 days incubation.

Elicitors	Observation of visible ascomata on plate of each media	
	MYPA	SDA
	appearance on plate	appearance on plate
Control	++	+
TTC	+	++
MPA	+++	++
TCZ	++	+
NYS 1 U	+	+
PHA 0.1 U	+++	+
LB 0.5 µg	+	+
LB 0.25 µg	+	+
JAS 0.05 µM	++	+
JAS 0.01 µM	++	+
HDACI IV 1 µM	N/O	N/O
HDACI IV 0.5 µM	N/O	N/O
HDACI IV 0.05 µM	N/O	N/O
HDACI II 0.8 µM	+	N/O
HDACI II 0.4 µM	N/O	N/O
HDACI II 0.2 µM	N/O	N/O

***** +++ visible ascomata cover entire colony, ++ visible ascomata abundance at the edge of the colony, + visible ascomata under dissection microscope, N/O no ascomata production.

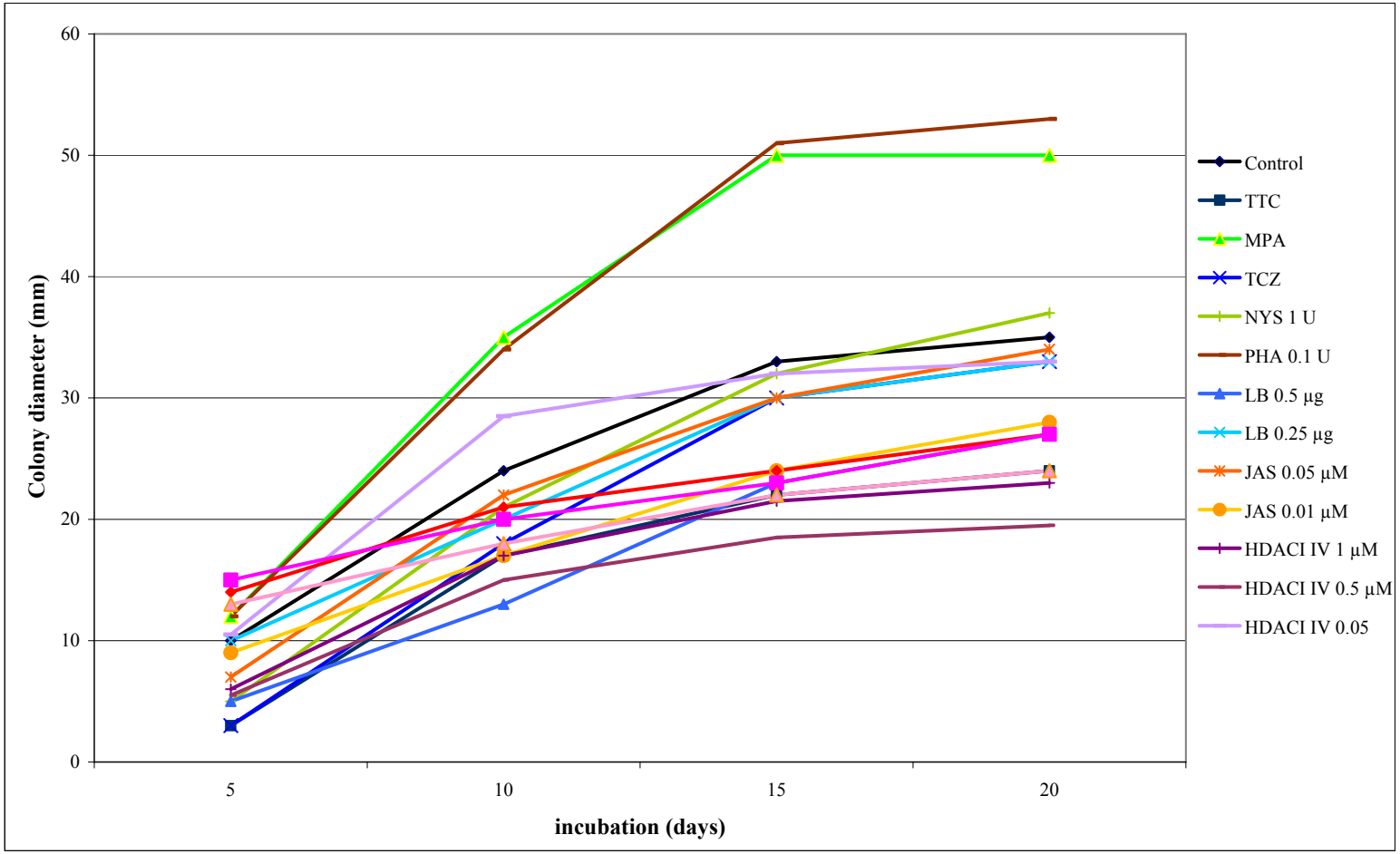


Figure 5.7: Effect of elicitors on growth of *P. zonatum* on MYPA medium at 20 °C.

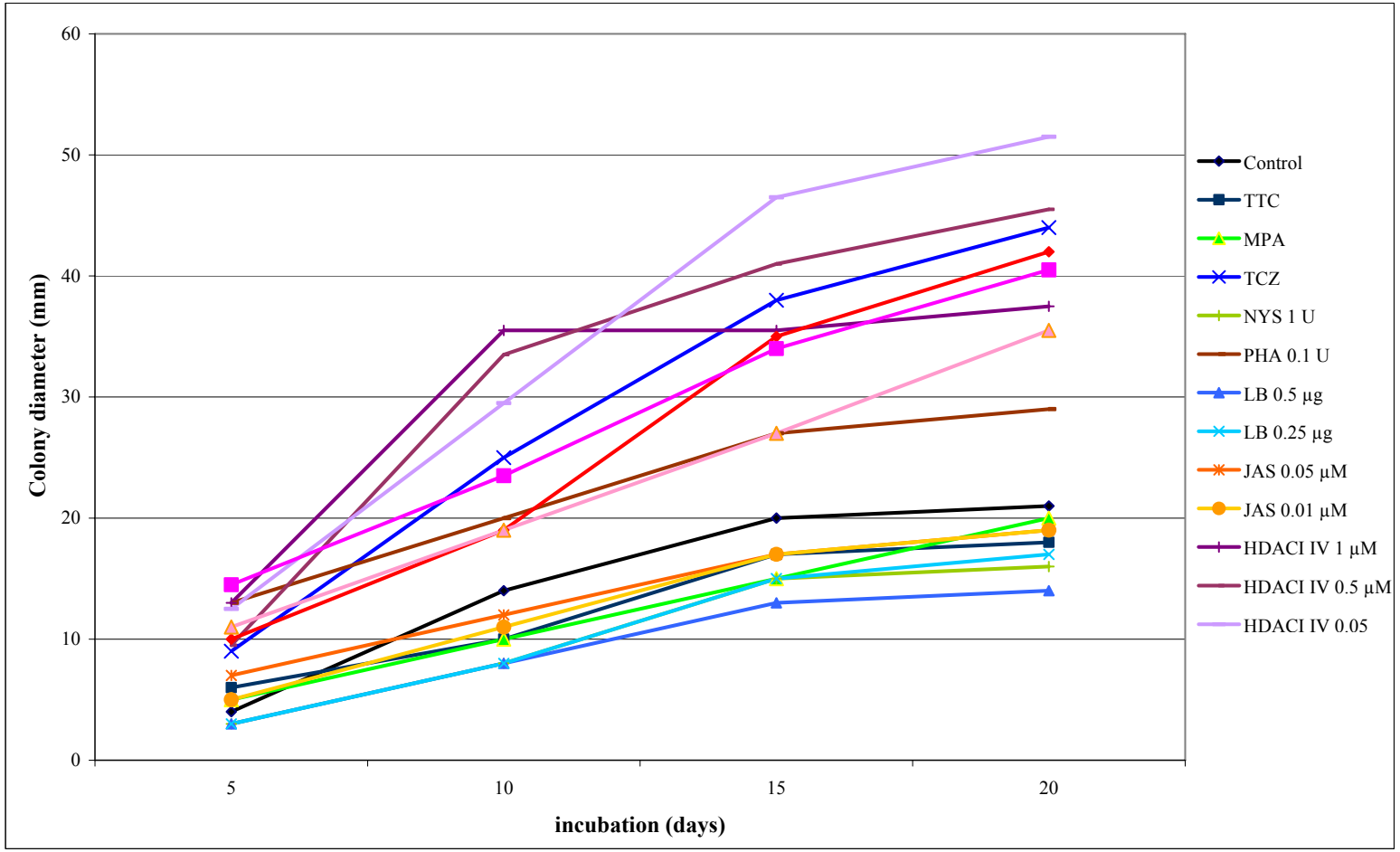


Figure 5.8: Effect of elicitors on growth of *P. zonatum* on SDA medium at 20 °C.

It was noted that no growth was observed in the presence of cycloheximide and nystatin (100 U and 10 U concentrations) in either media. Without the elicitors the growth of the fungus was greater on MYPA medium than SDA medium. The growth rate declined after 15 days incubation. Mycelial growth and ascomata production was enhanced with mycophenolic acid (MPA) and phalloidin (PHA) present in MYPA medium, but not enhanced when present in SDA medium. Growth and ascomata production was suppressed in the presence of histone deacetylase inhibitor II (HDACI II) and histone deacetylase inhibitor IV (HDACI IV) at any concentration on MYPA medium. It was noted that on SDA medium mycelial growth was enhanced by HDACI II and HDACI IV with the best mycelial growth seen with HDACI IV at 0.05 μM. Development of ascomata, however, was suppressed by HDACI II and HDACI IV.

5.5.2 The effect of elicitors on metabolite production

5.2.2.1 Cytotoxicity and HPLC screening

The initial extract (F7301) from *P. zonatum* (isolate 381_4@20.2) was screened for bioactivity and showed potent activity (>80% cells inhibition) against P388, *B. subtilis* and *C. albicans* in the quick screens, with subsequent determination of a P388 IC_{50} value of 1,035 ng/mL. The HPLC profile of the extract (**Figure 5.9**) showed two distinct peaks, named F7301-1 and F7301-2, which eluted at 16.8 and 19.7 minutes respectively. To determine the

bioactivity of each peak, an extract was subjected to HPLC microtitre plate screening with P388, *B. subtilis* and *C. albicans* (method described in **Chapter 2 section 2.7.3**). Results showed that peak F7301-1 was active against P388 cells, *B. subtilis* and *C. albicans*, while peak F7301-2 was active against P388 cells and *B. subtilis*, but not *C. albicans*. P388 IC_{50} values for peak F7301-1 and F7301-2 were determined as 1,837 and 9,837 ng/mL respectively.

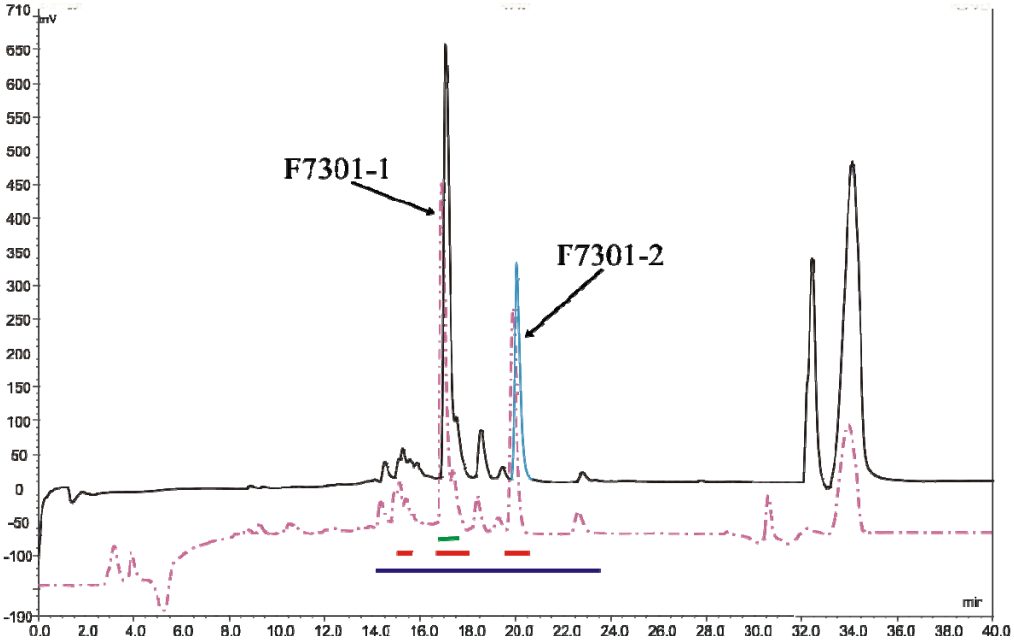


Figure 5.9: HPLC chromatogram of F7301 showing overlay of ELSD detection (solid) and the UV detection (pink-dash). The lines below UV detection chromatogram represent bioactive region(s) as tested against P388 cell (blue), *B. subtilis* (red) and *C. albicans* (green).

The cytotoxicities of the extracts from each elicitor treatment are given in **Table 5.5**. This isolate showed better IC_{50} values when it was grown on MYPA than SDA. No modulation in metabolite production was observed in the presence of elicitors. Nevertheless, an enhancement in metabolite

production was seen in the presence of elicitors. The growth of *P. zonatum* was not affected by HDACI II and HDACI IV, but the presence of these elicitors in both media at all concentrations suppressed metabolite production.

Table 5.5: Effect of elicitors on cytotoxicity of *P. zonatum* on MYPA and SDA media culture at 20 °C for 20 days.

Elicitors	Cytotoxicity on both media			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9210	1,035	F9225	3,232
TTC	F9211	1,500	F9226	2,954
MPA	F9212	1,035	F9227	>12,500
TCZ	F9213	1,483	F9228	>12,500
NYS 1 U	F9216	1,483	F9231	2,256
PHA 0.1 U	F9220	1,035	F9235	>12,500
LB 0.5 µg	F9221	1,500	F9236	1,622
LB 0.25 µg	F9222	1,066	F9237	1,035
JAS 0.05 µM	F9223	1,355	F9238	1,622
JAS 0.01 µM	F9224	1,622	F9239	>12,500
HDACI IV 1 µM	F9421	5,542	F9428	>12,500
HDACI IV 0.5 µM	F9423	3,959	F9430	>12,500
HDACI IV 0.05 µM	F9425	4,916	F9432	>12,500
HDACI II 0.8 µM	F9518	11,722	F9524	>12,500
HDACI II 0.4 µM	F9520	8,687	F9526	>12,500
HDACI II 0.2 µM	F9522	4,916	F9528	>12,500

* P388 IC_{50} activity >12,500 ng/mL was considered to be inactive.

The ELSD traces of all 15 elicitors and control from MYPA (**Figure 5.10 a-b**) and SDA (**Figure 5.11 a-b**) showed the presence of compoundsF7301-1 and F7301-2.

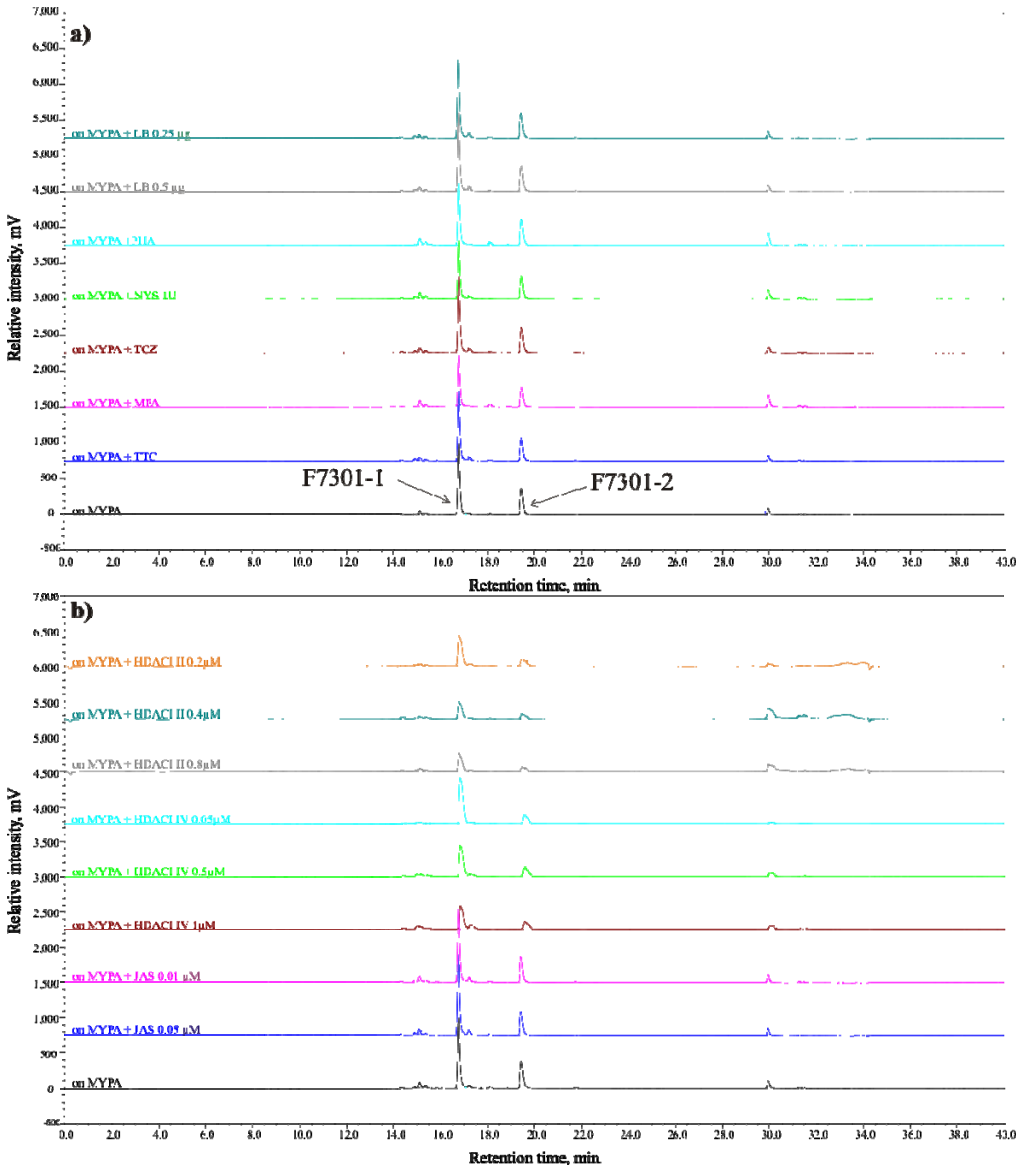


Figure 5.10 (a-b): HPLC traces of extracts from *P. zonatum* cultured on MYPA medium at 20°C for 20 days with addition of different elicitors in the medium.

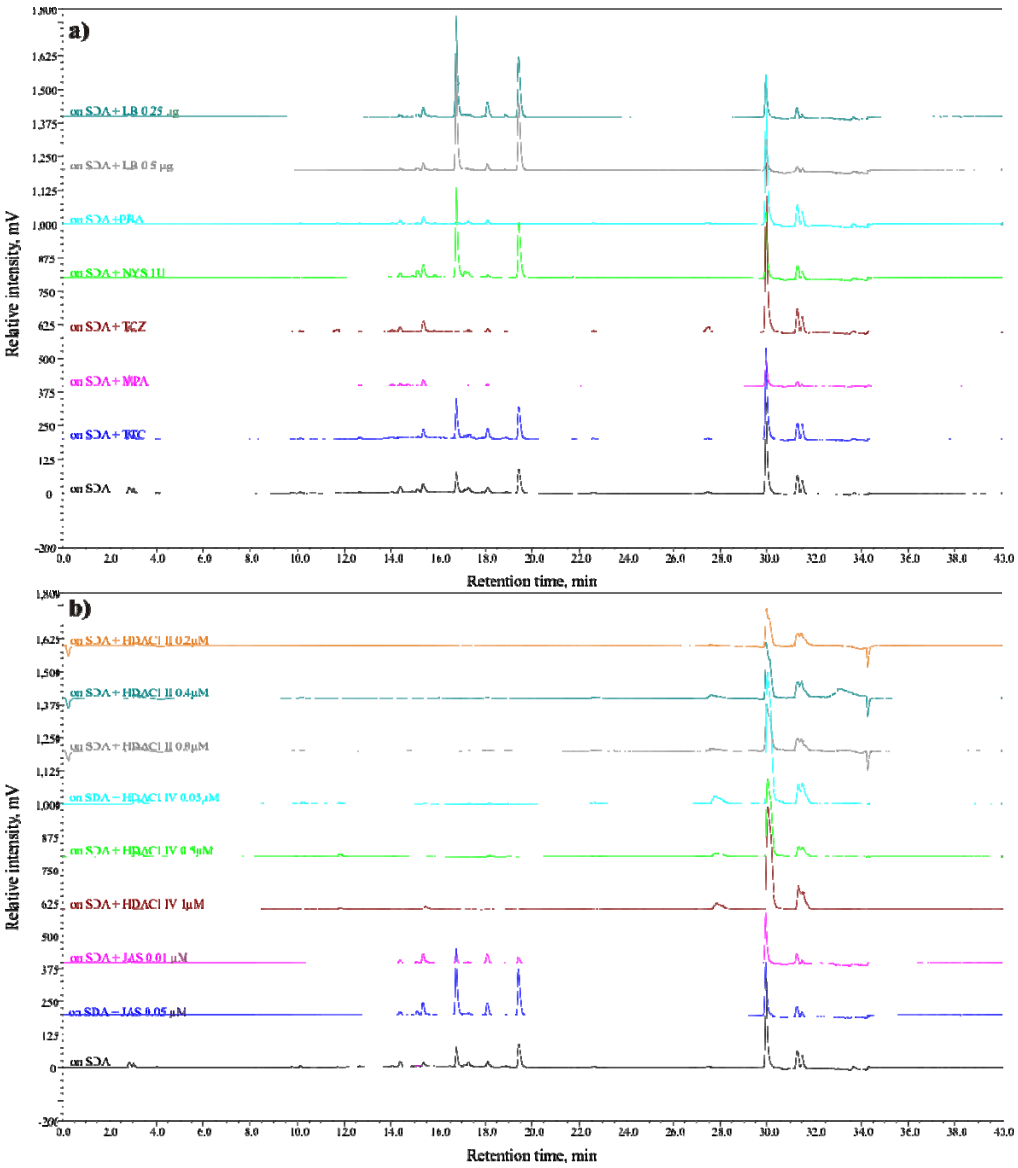


Figure 5.11 (a-b): HPLC traces of extracts from *P. zonatum* cultured on SDA medium at 20°C for 20 days with addition of different elicitors in the medium.

5.6 STRUCTURAL ELUCIDATION OF THE COMPOUNDS PRODUCED BY *P. zonatum*

The extracted UV spectra of F7301-1 and F7301-2 exhibited multiple UV absorption maxima at 202, 223 and 282 nm (**Figure 5.12**), suggesting that these two compounds were structurally related compounds. A search in the HPLC-UV/Rt library database for a compound with similar UV maxima showed that F7301-1 and F7301-2 could be related to either the cytochalasin, or the chaetoglobosin family of compounds (**Figure 5.12**). A comparison between cytochalasin and chaetoglobosin structures showed that many of the compounds in these families share the basic structure of an indole subunit. The UV absorption maxima of F7301-1 and F7301-2 have UV chromophores similar to those of an indole system, as reported by Andonovski and Stojkovtić (2000).

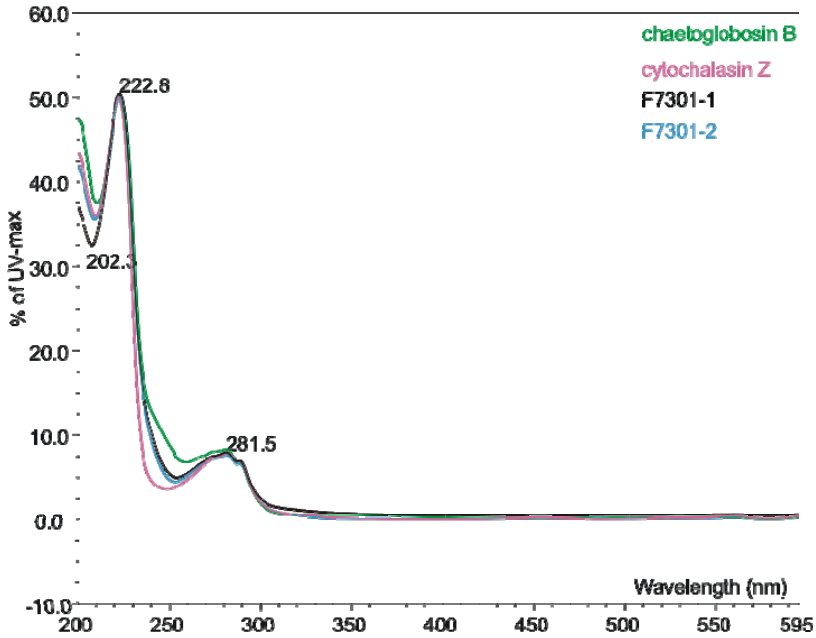


Figure 5.12: A comparison of UV absorption maxima of F7301-1 (black), F7301-2 (blue), chaetoglobosin B (green) and cytochalasin Z (pink).

The positive ion Electrospray Ionization mass spectrum (ESIMS) of compound F7301-1 suggested a molecular mass of 473 amu ($[M+H]^+$) (**Figure 5.13a**) and 457 amu ($[M+H]^+$) for compound F7301-2 (**Figure 5.13b**).

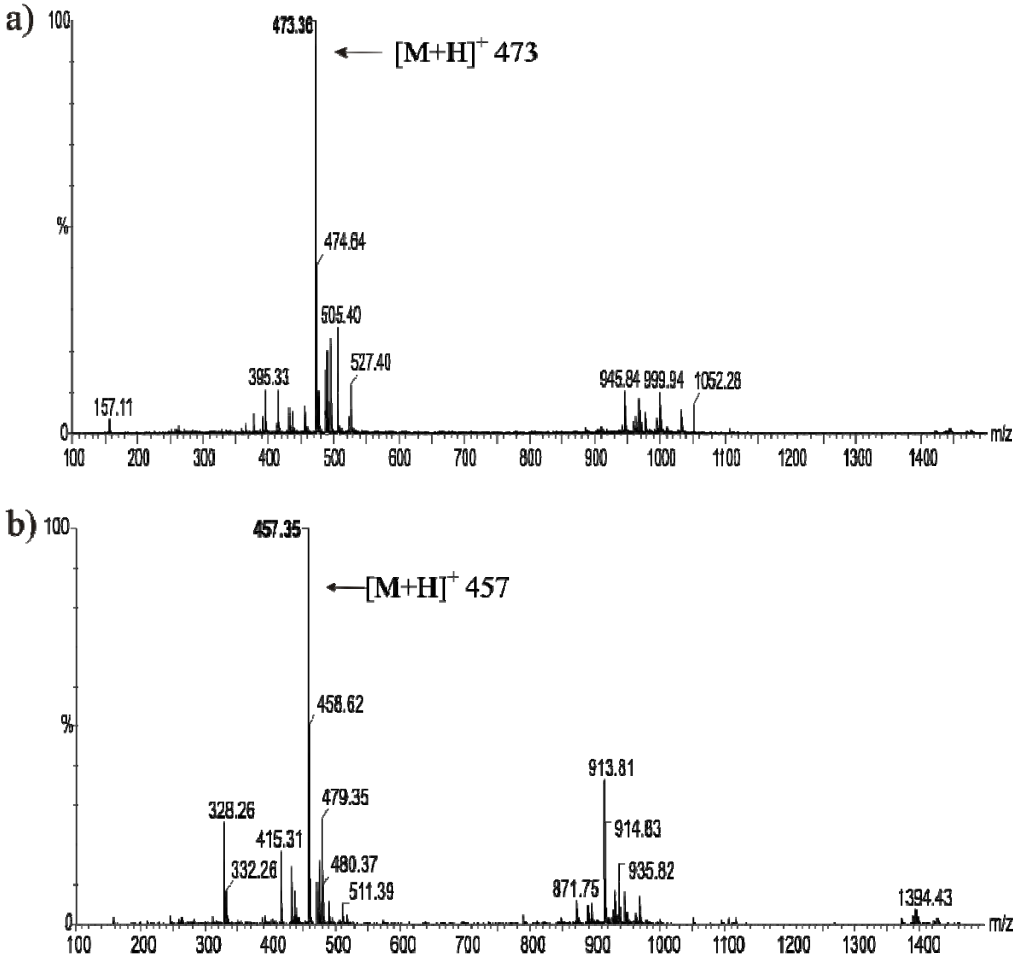


Figure 5.13: **a)** ESIMS spectrum of compound F7301-1; **b)**ESIMS spectrum of compound F7301-2

A search of the AntiMarin database using these molecular masses and an indole group substructure resulted in no hits with any compounds within the database for the compound in the range *m/z* 472-474 amu. However, for the compound F7301-2 (*m/z* 457-458 amu) there were four hits in the AntiMArin

database. However, none of these hits were related to either the cytochalasin or chaetoglobosin structure class. Both compounds were therefore investigated further to establish structures for these metabolites.

5.6.1 Fraction F7301-1

The ^{1}H NMR spectrum of F7301-1 (**Figure 5.14**) showed the presence of one singlet methyl group at δ_H 1.21 and two doublet methyl groups at δ_H 0.62 and 1.15. The aromatic region of the ^{1}H NMR spectrum exhibited two doublets at δ_H 7.34 and 7.63, two triplets at δ_H 7.04 and 7.09, and one singlet at δ_H 7.15. These two doublets and two triplets are characteristic of 1, 2-disubstitued aromatic ring of an indole system while the singlet represents a proton adjacent to the NH group in the indole system (H-2′) (**Figure 5.15**). The signals at δ_H 5.22, 6.21, 6.27 and 7.86 represent olefinic hydrogens in the compound. A search in the AntiMarin database using the above criteria revealed thirteen hits, but none of them matched with the observed molecular weight (472) of the compound.

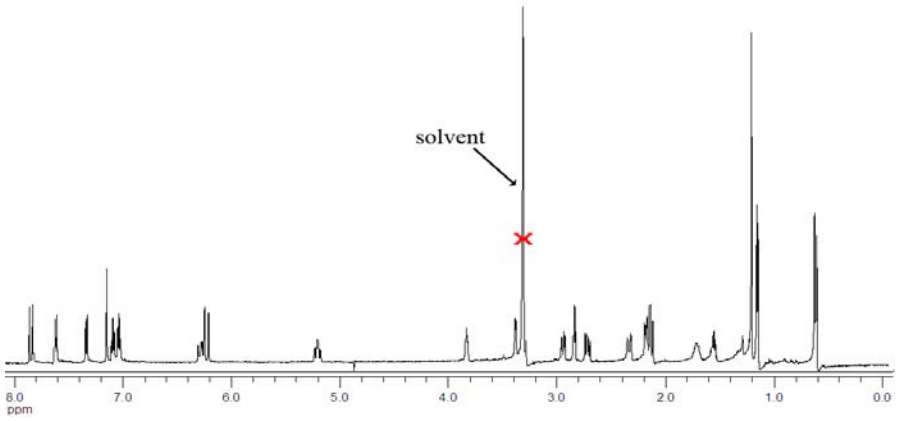


Figure 5.14: ^{1}H NMR spectrum of F7301-1, 34 µg in 6 µL CD_3OD, recorded 500 MHz, 100sec.

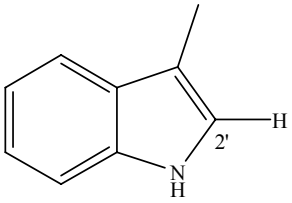


Figure 5.15: Indole system

A series of 2D NMR experiments; Homonuclear Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) were performed to generate the data for structural elucidation.

The HSQC experiment (**Figure 5.16**) disclosed the presence of three methylene (CH_2) groups in the compound at δ_C 33.5, 43.9 and 47.1. From the COSY and HMBC experiments (**Figure 5.17** and **5.18**) three different fragments were deduced.

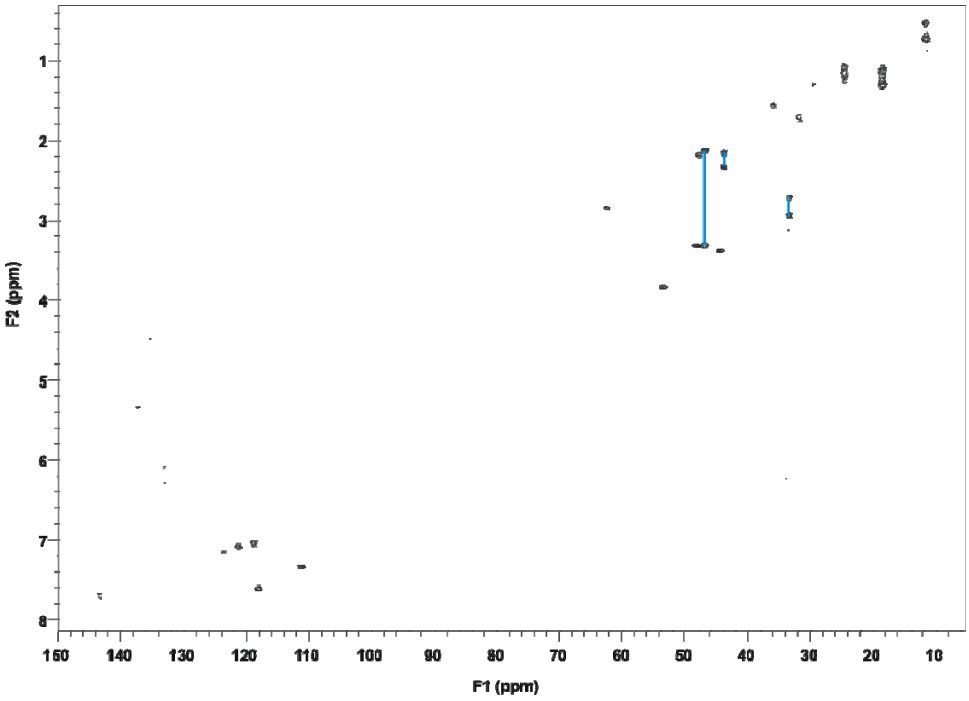


Figure 5.16: HSQC NMR spectrum of F7301-1 in CD_3OD (blue lines represent CH_2 group).

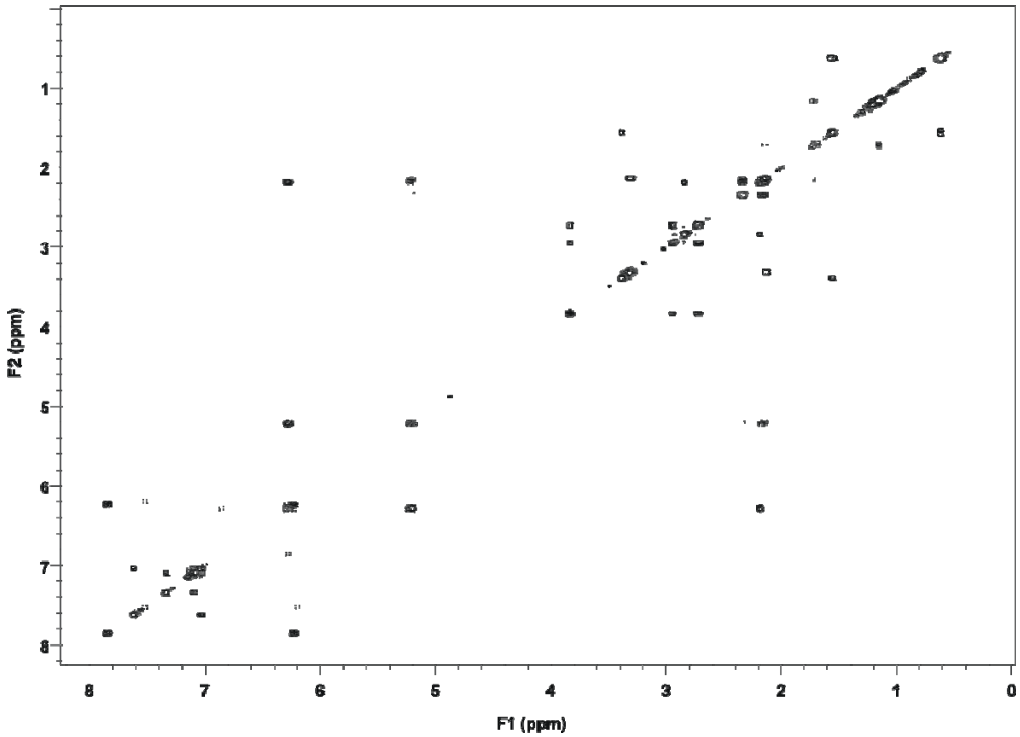


Figure 5.17: COSY NMR spectrum of F7301-1 in CD_3OD.

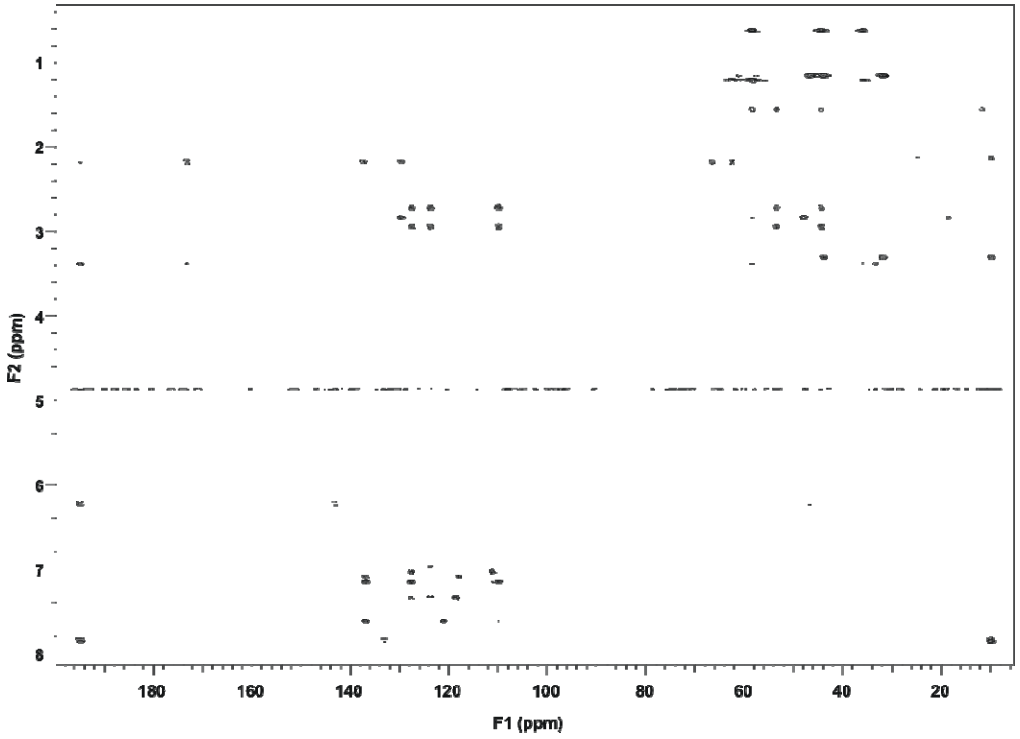


Figure 5.18: HMBC NMR spectrum of F7301-1 in CD_3OD.

The first fragment consisted of an assignment of protons and carbons in an indole system (**Figure 5.19**). In the COSY NMR spectrum the proton at δ_H 7.34 was coupled to δ_H 7.09, which was in turn was coupled to a signal at δ_H 7.04. This proton was, in turn, coupled to δ_H 7.63. The carbon assignments for these protonated carbons were assigned from the HSQC NMR spectrum ($^1J_{CH}$). From the HMBC spectrum, a proton at δ_H 7.04, H-6′ was correlated to carbons at δ_C 111.3, C-8′ ($^3J_{CH}$) and δ_C 127.6. The carbon at δ_C 127.6 was assumed to be a carbon at position 4′ because the HSQC experiment suggested the two vicinal carbon of C-6′ (C-5′ and C-7′) were assigned different carbon chemical shifts. The C-4′ chemical shift was then confirmed by $^2J_{CH}$ coupling from H-5′ and $^3J_{CH}$ coupling from H-2′ and H-8′ (**Figure 5.19**). From the long range connectivities of H-2′ it was assumed that the carbon chemical shifts at C-3′ and C-9′ could be assigned as δ_C 109.9 and 136.9. Furthermore, H-7′ showed a long range connectivity ($^3J_{CH}$) to carbon at δ_C 136.9, thus suggesting its assignment to the C-9′ position. The carbon at δ_C 109.9 was confirmed to be assigned to C-3′ by the $^3J_{CH}$ coupling from H-5′.

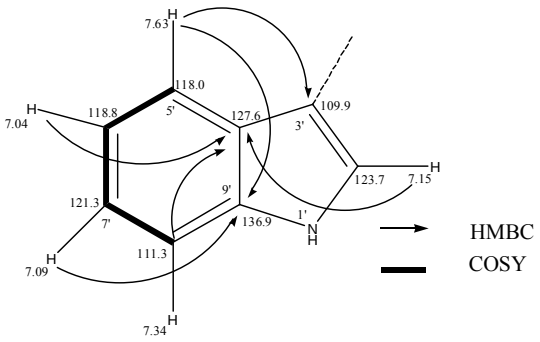


Figure 5.19: Indole spin system of F7301-1 obtained from COSY, HSQC and HMBC NMR spectral data.

Fragment 2; from the COSY NMR spectrum, protons at δ_H 0.62 were coupled to δ_H 1.53 which in turn was coupled to a signal at δ_H 3.38 (**Figure 5.20a**). The proton resonance at δ_H 3.83 was coupled to the methylene protons at δ_H 2.71 and 2.94 (**Figure 5.20b**). The proton resonance at δ_H 2.19 was coupled to protons at δ_H 2.84 and 6.27 while the olefinic proton at δ_H 6.27 was further coupled to another olefinic proton at δ_H 5.22 which was then in turn coupled to methylene protons at δ_H 2.14 and 2.32 (**Figure 5.20c).**

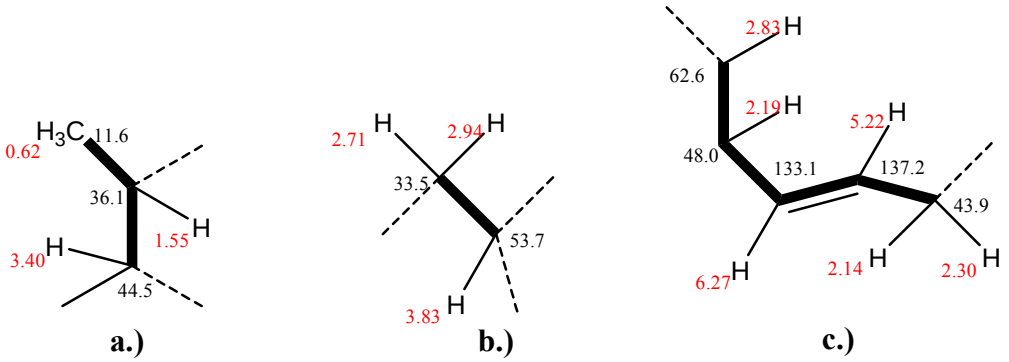


Figure 5.20: Spin systems of F7301-1 obtained by COSY (solid line) and HSQC.

From the HMBC NMR spectrum, the methyl protons at δ_H 0.62 showed a $^2J_{CH}$ correlation to a methine carbon at δ_C 36.2, and $^3J_{CH}$ correlations to a methine carbon at δ_C 44.5 and a quaternary carbon at δ_C 58.4, giving the substructure as depicted in **Figure 5.21a**. A singlet methyl signal at δ_H 1.21 showed correlations to δ_C 36.2, 58.4 and 62.7. This placed the methyl signal next to the quaternary carbon at δ_C 58.4 (**Figure 5.21b**). A proton at δ_H 2.84 (see **Figure 5.20c**) showed correlations to carbons at δ_C 18.5, 48.0, 58.4 and 133.1 thus joining fragment **5.20c** and **5.21b**, to give the substructure depicted in **Figure 5.21c**.

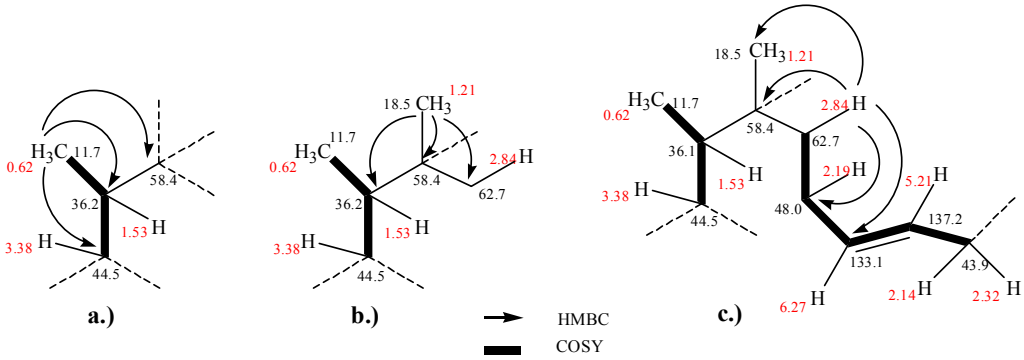


Figure 5.21: HMBC correlations observed for the proton at **a)** δ_H 0.62; **b)** δ_H 1.21; and **c)** δ_H 2.84.

The chemical shift of both the quaternary carbon at δ_C 58.4 and the tertiary carbon at δ_C 62.7 indicated the presence of an oxygen atom, therefore, the presence of a trisubstituted epoxide was suggested (**Figure 5.22**).

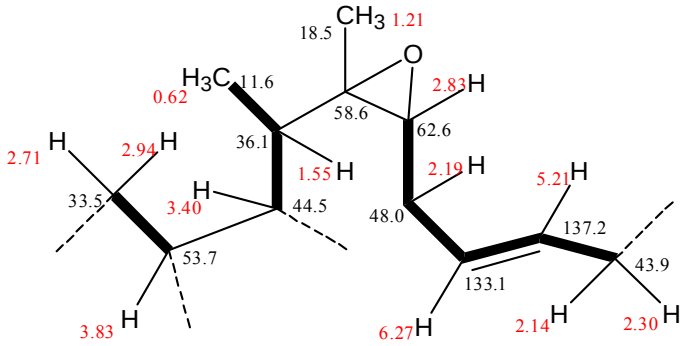


Figure 5.22: Trisubstituted epoxide fragment

The proton at δ_H 2.94 showed a $^3J_{CH}$ correlation to a methine carbon at δ_C 44.5 (see **Figure 5.22**) as well as showed $^2J_{CH}$ and $^3J_{CH}$ correlations to carbons in the indole system at δ_C 109.9 and 123.7 (**Figure 5.19**) respectively. Thus the indole system could be connected the fragment given in **Figure 5.22**, to give a larger fragment as shown in **Figure 5.23**.

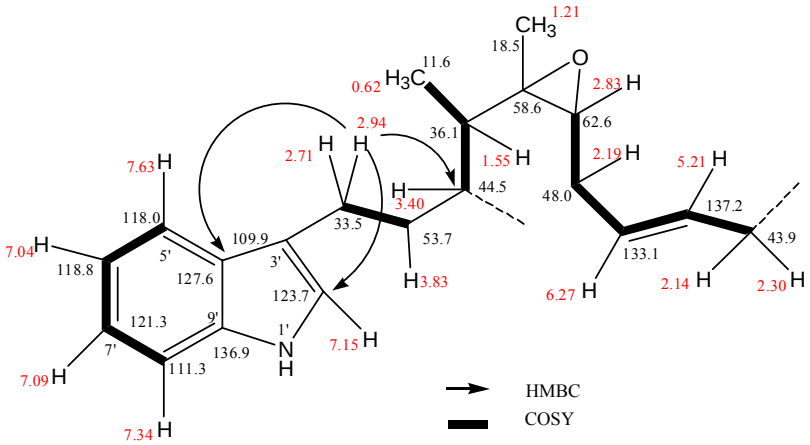


Figure 5.23: HMBC correlations observed for the proton at δ_H 2.94.

The third fragment was assembled from the remaining substructure units as revealed in the COSY experiment. This showed that the methyl proton at δ_H 1.15 was coupled to a proton at δ_H 1.72 (**Figure 5.24a**) and that the olefinic protons at δ_H 6.21 and 7.86 were coupled to each other (**Figure5.24b**). The HSQC NMR experiment showed that the resonances at δ_H 2.12 and 3.31 were part of the same methylene group (**Figure 5.24c**).

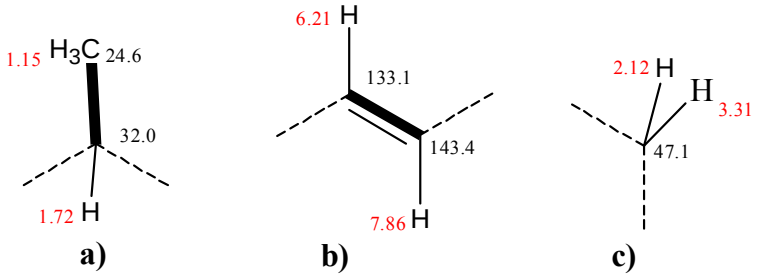


Figure 5.24: Spin systems of F7301-1 obtained by COSY (bold line) and HSQC.

From the HMBC experiment, the methylene protons at δ_H 2.12 and 3.31 showed correlations to the carbonyl carbon at δ_C 204.9 as well as to the methine carbon at δ_C 32.0 (**Figure 5.25a**). The proton at δ_H 7.86 showed correlations to

the carbonyl carbons at δ_C 204.9 and δ_C 195.0. The proton at δ_H 6.21 also showed a correlation to the carbonyl carbon at 195.0. Therefore, the carbonyl carbon at δ_C 195.0 was assigned as being adjacent to the olefinic carbon at δ_C 133.1 (**Figure 5.25b**) thereby defining the third unit.

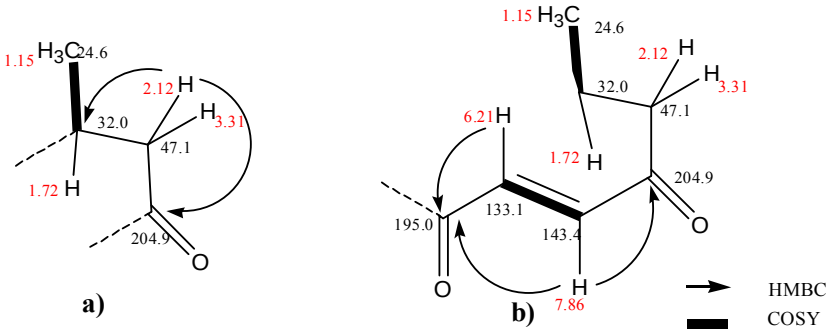


Figure 5.25: HMBC correlations observed for the protons **a)** δ_H 2.12 and 3.31; **b)** δ_H 6.21 and 7.86

As the methyl protons at δ_H 1.15 showed a $^3J_{CH}$ correlation to the methylene carbon at δ_C 43.9, and the proton at δ_H 5.21 showed a $^3J_{CH}$ correlation to the carbon at δ_C 32.0, the structure fragment from **Figure 5.23** could be linked with the structure fragment given in **Figure 5.25b** to give the structure fragment shown in **Figure 5.26.**

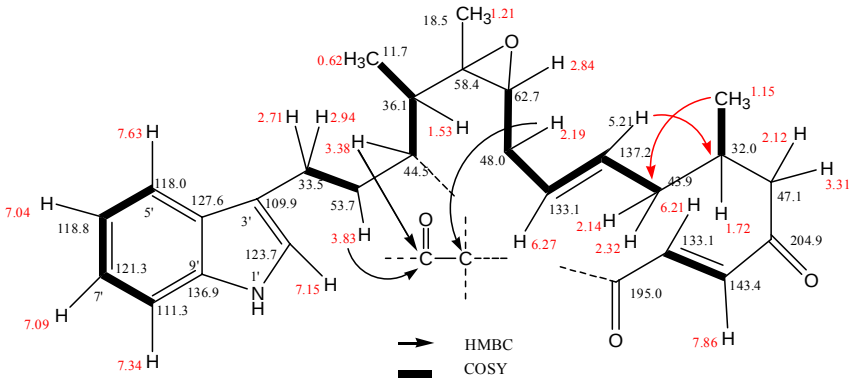
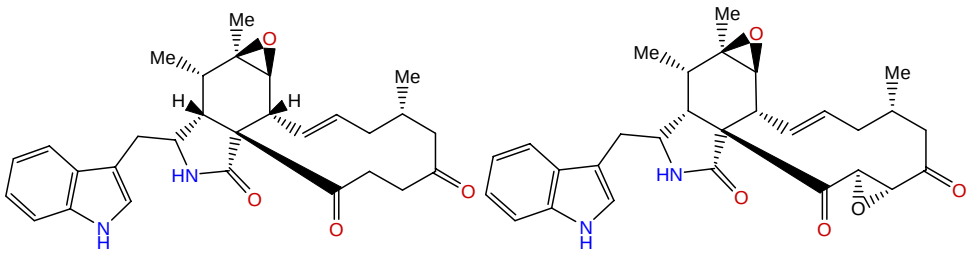


Figure 5.26: HMBC correlations observed for protons at δ_H 1.15, 5.21 (red arrows), 2.19, 3.38 and 3.83 (black arrows) .

The protons at δ_H 2.19, 3.38 and 3.83 all correlated with the third carbonyl group at δ_C 173.3 (**Figure 5.26**) , while the δ_H 2.19 proton was also correlated to a quaternary carbon at δ_C 66.9 (see **Figure 5.26**). The molecular mass of compound F7301-1 was found to be 472 amu, while the molecular mass of the structure fragment shown in **Figure 5.26** with an additional carbonyl group and a quaternary carbon requires 457 amu. This leaves 15 amu unaccounted for in the molecular mass, which could be assigned to an NH group. From previous searching of the AntiMarin database, compound F7301-1 closely resembled the NMR data reported for cytochalasin Z, which is closely related to cytochalasin G (**Figure 5.27**) as reported by Feng *et. al*. (2002). This suggested that the remaining NH, carbonyl carbon and quaternary carbon could be included as a lactam ring as seen in both the structures of cytochalasin G and Z.



a) Cytochalasin G **b)** Cytochalasin Z

Figure 5.27: cytochalasin G and cytochalasin Z.

A comparision of the NMR data with the literature data of cytochalasin Z together with the data derived in the preceeding discussion led to the proposal of the planar structure of F7301-1 as shown in **Figure 5.28**. As the literature NMR data for cytochalasin G and Z was obtined in $CDCl_3$ the ^1H NMR was reacquired for F7301-1 in $CDCl_3$ to permit a direct comparison as shown in **Table 5.6**.

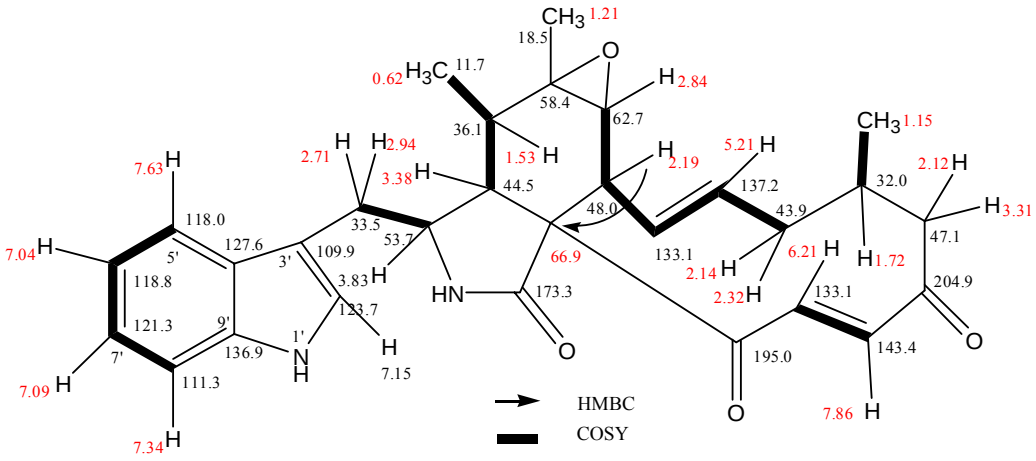


Figure 5.28: Planar structure of compound F7301-1

Table 5.6: ^{1}H NMR data of F7301-1 and Cytochalasin G and Z (500 MHz in $CDCl_3$)

Position	F7301-1 run in CD_3OD					F7301-1 run in $CDCl_3$				Cytochalasin Z				Cytochalasin G			
	δ_H	splitting	*J* Hz			δ_H	splitting	*J* Hz		δ_H	splitting	*J* Hz		δ_H	splitting	*J* Hz	
1	-					-	-			-	-			-	-		
2						5.91	br s			6.65	br s			5.98	br s		
3	3.83	ddd	2.2	5.0	7.5	3.84	m			3.84	m			3.80	t	5.5	
*4	3.38	dd	2.2	5.7		3.34	dd	6.6	12.3	3.21	dd	5.5	2.0	2.87	br d	5.0	
5	1.53	dt	7.3	5.9		1.73	t	6.8		1.77	t	6.5		1.89	br s		
6	-					-	-			-	-						
7	2.84	d	5.5			2.87	d	5.5		2.81	d	5.5		2.82	d	5.5	
8	2.19	m				2.18	m			2.12	dd	10.0	5.5	2.10	dd	10.5	6.5
9	-					-	-			-	-						
10a	2.94	dd	4.9	13.8		2.98	dd	3.3	13.8	2.95	dd	4.0	14.0	2.89	dd	14.0	4.5
10b	2.71	dd	8.3	14.1		2.73	dd	9.2	14.3	2.72	dd	8.5	14.0	2.64	dd	14.5	6.5
11	0.62	d	7.3			1.15	d	7.5		1.22	d	7.5		1.09	d	7.0	
12	1.21	s				1.27	s			1.24	s			1.20	s		
13	6.27	dd	11.4	15.6		6.26	dd	11.4	15.6	6.36	dd	15.0	10.0	6.14	dd	15.5	10.0
14	5.22	ddd	4.0	11.0	15.2	5.18	ddd	3.3	11.0	5.40	dtd	11.5	3.5	5.05	dtd	11.5	3.5
15a	2.32	d	13.4			2.34	d	14.1		2.33	m			2.25	m		
15b	2.14	m				2.16	m			1.9-2.0	m			1.6-1.7	m		
16	1.72	m				1.64	m			2.25-2.30	m			2.3-2.4	m		
17a	3.31	m				3.47	br s			2.60	dd	15.5	4.0	2.4-2.5	m		
17b	2.12	m				2.14	m			2.36-2.42	m			1.86	t	3.5	
18	-					-	-			-	-	-					
19	7.86	d	16.5			7.96	d	16.5		4.57	d	2.0		2.6-2.7	m		
19b														2.3-2.4	m		
20	6.21	s				6.30	d	16.3		3.42	d	2.0		3.42	dd	17.0	12.5
20b														1.6-1.7	m		
21	-					-	-			-		-					
22	1.15	d	7.0			1.18	d	7.0		1.17	d	6.5		0.95	d	6.5	
1'										8.59	br s			8.31	br s		
2'	7.15	s				7.07	s			7.04	s			6.97	d	2.5	
3'	-					-	-			-		-					
4'	-					-	-			-		-					
5'	7.63	d	7.9			7.55	d	7.9		7.53	d	8.0		7.49	d	8.0	
6'	7.04	t	7.2			7.17	t	7.5		7.16	t	6.5		7.15	t	8.0	
7'	7.09	t	7			7.23	t	7.3		7.24	t	7.0		7.20	t	8.0	
8'	7.34	d	81			7.40	d	8.3		7.46	d	8.5		7.35	d	8.5	
9'	-					-				-		-					

Note: *4 indicate stereocentre; chemical shifts in red colour indicate the positions used to assign the stereochemistry of F7301-1.

The tabulation of the proton chemical shifts of cytochalasin G, cytochalasin Z and F7301-1 **(Table 5.6)** in $CDCl_3$ shows the close structural identity between these structures. The major differences in the ^1H values for these three compounds were centred on H-19 and H-20. In cytochalasin G these protons were methylenes, but in cytochalasin Z, H-19 and H20 (δ_H 4.57 and 3.42 respectively) were part of an epoxide system. In F7301-1 H-19 and H-20 appeared at δ_H 7.96 and 6.30 respectively and were assigned as olefinic protons. This comparison supports the proposal that F7301-1 is similar to cytochalasin Z except for the replacement of the epoxide with a double bond at positions 19 and 20. Furthermore, the coupling constant for H-19/H-20 was 16.5 Hz indicating a *trans* disposition of protons across the double bond.

Previous studies have suggested that the essential elements of the cytochalasin skeleton have the same stereochemistry, viz., the *cis*-sterochemistry across the 5/6 ring junction and the *trans*-sterochemistry of the macro cyclic ring (Natori and Yahara, 1991).

The stereochemistry of F7301-1 was determined as shown in **Figure 5.29** by comparing the proton chemical shifts and coupling constants of F7301-1 with the literature data of cytochalasin Z and cytochalasin G as shown in red in **Table 5.6**.

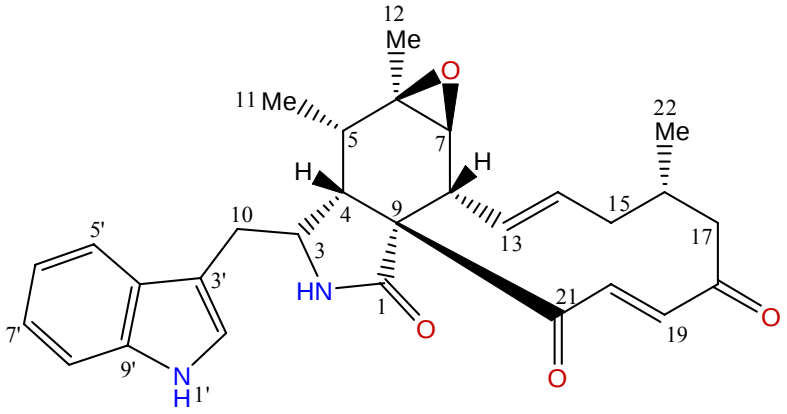


Figure 5.29: Structure of F7301-1

This comparison supports the structure proposed in **Figure 5.29** except that the proton at position 4 of F7301-1 in $CDCl_3$ has different coupling constants compared with the reported data. However, the nuclear overhauser effect (NOE) NMR experiments which were performed in CD_3OD were in agreement with a configuration of H-4 as proposed in **Figure 5.29** as irradiation of the H-3, H-4 and H-11 protons gave the expected enhancements of H-3, H-4 and H-11 as listed in **Table 5.7**.

Table 5.7: NOEs observed in F7301-1

Irradiation at	NOE enhancement
H-3	H-11, H-12
H-4	H-5
H-11	H-12

5.6.2 Fraction F7301-2

The general features of the ^{1}H NMR spectrum of F7301-2 (**Figure 5.30**) were found to closely resemble those of F7301-1, except in the region of the six-membered ring. A proton resonance at δ_H 2.84, H-7 in F7301-1 was replaced by an olefinic proton at δ_H 5.40.

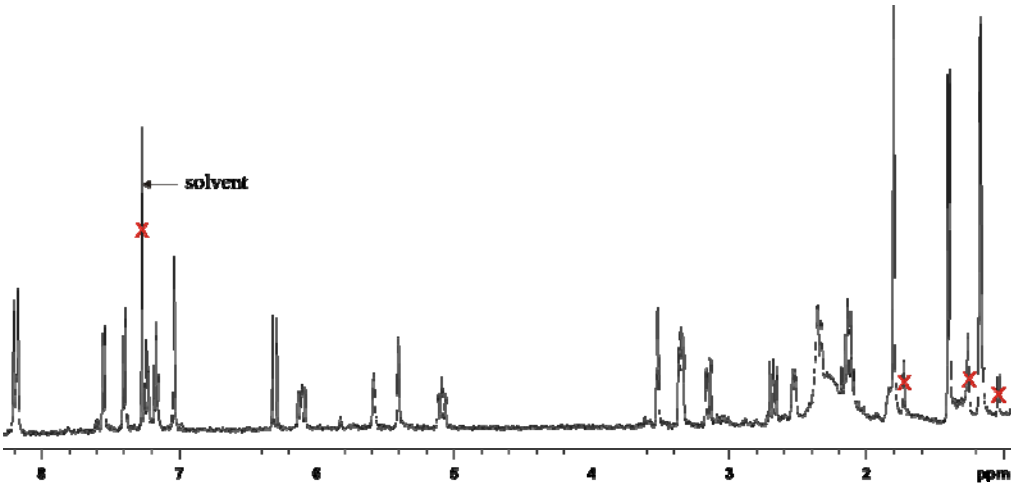


Figure 5.30: ^{1}H NMR spectrum of F7301-2, 25 µg in 6 µL $CDCl_3$, recorded at 500 MHz, 100sec, (crosses indicate impurities).

Furthermore, the difference in the molecular mass of compound F7301-1 compared to F3301-2 was 16 amu, immediately suggesting the replacement of an epoxide with a double bond at positions 6 and 7. A series of 2D NMR experiments (COSY, HSQC and HMBC) were conducted and compared with the data for compound F7301-1 (showed in **Table 5.8**) in order to confirm this replacement as well to ptovide complete assignment of all protons and carbons.

Table 5.8: Summary table of NMR experiments of F7301-1 and F7301-2

Position	F 7301-1																	F 7301-2 run in $CDCl_3$												
	run in CD_3OD					run in $CDCl_3$				run in CD_3OD																				
	δ_H	splitting	*J* Hz			δ_H	splitting	*J* Hz		COSY		δ_C	multiple bond correlation						δ_H	splitting	*J* Hz			COSY			δ_C	multiple bond correlation			
1	-					-						173.3							-								-				
2						5.91	br s												5.58	s											
3	3.83	ddd	2.2	5.0	7.5	3.84	m			2.70	2.95	53.7	36.0	173.2					3.34	q	7.1	4.8	11.8	2.67	3.49		54.6				
4	3.38	dd	2.2	5.7		3.34	dd	6.6	12.3	1.53		44.5	33.6	36.2	58.6	66.7	173.4	195.2	3.51	t	4.5			2.33	3.34		47.5	34.5	54.3	68.2	196.1
5	1.53	m				1.73	m			0.62	3.38	36.2	11.7	44.3	53.7	58.6			2.35	d	2.4			1.38	3.51		34.7				
6	-					-						58.4							-								141.5				
7	2.84	d	5.5			2.87	d	5.5		2.19		62.7	18.5	48.0	58.4	133.1			5.40	d				1.78	2.54		124.8				
8	2.19	m				2.18	m			2.84	6.27	48.0	62.7	66.9	133.1	137.4	173.4	195.2	2.52	d	10.5			1.81	5.39	6.08	46.9				
9	-					-						66.9							-								68.2				
10a	2.94	dd	8.3	14.1		2.98	dd	3.3	13.8	2.71	3.80	33.5	44.5	53.6	109.9	123.8	127.8		3.15	dd	3.1		14.4	3.34	3.67		35.1				
10b	2.71	dd	4.9	13.8		2.73	dd	9.2	14.3	3.80	2.94	33.5	44.5	53.6	109.9	123.7	127.8		2.66	q	10.5	3.7	14.3	3.15	3.34		35.1	111.9			
11	0.62	d	7.3			1.15	d	7.5		1.53		11.7	36.2	44.3	58.6				1.39	d	7.3			2.35			14.5	34.6	47.1	141.6	
12	1.21	s				1.27	s					18.5	36.0	58.4	62.7				1.77	s				5.38			20.6	34.6	124.8	141.6	
13	6.27	t				6.26	m			2.19	5.21	133.1	47.3	143.0	195.2				6.08	m				2.51	5.09						
14	5.22	ddd	4.0	11.0	15.2	5.18	ddd	3.3	11.0	2.19	6.27	137.2							5.09	m	3.3	11.5	14.8	2.15	6.09		130.7				
15a	2.32	d	13.4			2.34	d	14.1		2.19	2.95	43.9							2.32	d	2.4			2.13			43.7				
15b	2.14	m				2.16	m			5.22	1.72	43.9	31.9	24.9					2.13	d				2.34	5.09		43.7	130.7			
16	1.72	m				1.64	m			1.16	2.14	32.0							1.81	m				1.16	2.12	2.54	32.8	204.9			
17a	3.31	m				3.47	br s			2.12		47.1	32.0	44.0	204.9				3.34	q	7.1	4.8	11.8	2.11			46.2	204.9	32.4	43.3	
17b	2.12	m				2.14	m			3.31		47.1	32.0	204.9					2.11	m				1.83	3.34		46.2	204.9	25.7	33.0	
18	-					-						204.9							-								204.9				
19	7.86	d	16.5			7.96	d	16.5		6.21		143.4	133.1	195.0	204.9				8.18	d	16.5			6.3			76.15	132.9	196.1	204.9	
20	6.21	s				6.30	d	16.3		7.86		133.1	143.5	195.0					6.29	d	16.7			8.2			132.9	45.9			
21	-					-						195.0							-								196.1				
22	1.15	d	7.0			1.18	d	7.0		1.72		24.6	31.9	43.7	47.3				1.16	d	6.9			1.8			26.02	32.9	43.3	45.9	
1'																											-				
2'	7.15	s				7.07	s					123.7	109.9	127.8	136.9				7.03	s							122.9	112.0	126.9	136.6	
3'	-					-						109.9							-								112				
4'	-					-						127.6							-								136.6				
5'	7.63	d	7.9			7.40	d	7.9		7.04		118.0	109.9	121.3	136.9				7.39	d	8.1			7.2			111.9	126.9			
6'	7.04	t	7.2			7.17	t	7.5		7.61	7.09	118.2	111.4	127.6					7.22	t	7.3			7.4			122.5	118.5	136.6		
7'	7.09	t	7			7.23	t	7.3		7.34	7.04	121.3	118.2	136.9					7.16	t	7.2			7.55			120.8	111.7	126.9		
8'	7.34	d	81			7.55	d	8.26		7.09		111.3	118.2	123.5	127.6				7.54	d	8.0			7.16			119.0	122.5	136.6		
9'	-					-						136.9							-								126.9				

From comparison of the data in **Table 5.8**, the proton chemical shifts of both compounds are almost identical. The major points of difference are noted in red. The main structure features that had been found in compound F7301-1 such as, one singlet methyl (C-12); two doublet methyl groups(C-11 and 22), an indole system, an alkene (Δ19), were also found in compound F7301-2.

The major diffferences in the ^{1}H δ values for these two compounds were to be found centred on C-7. It was established or concluded that the proton at δ_H 2.84 in 1 was replaced with an olefinic proton at δ_H 5.40 in F7301-2. Small differences in the proton chemical shifts in the six-membered ring of the two compounds were also noted, confirming the change of functional group in this region.

The COSY NMR spectrum of F7301-2 showed that the olefinic proton at δ_H 5.40 was coupled to δ_H 2.52, and vice-versa. Furthermore, the HMBC experiment revealed that the methyl protons at δ_H 1.77 had a $^{3}J_{CH}$ correlation to δ_C 124.8 (**Figure 5.31**) once again confirming the interchange of an epoxide functionality with an alkene at positions 6 and 7. The planar structure of compound F7301-2 is depicted in **Figure 5.31**.

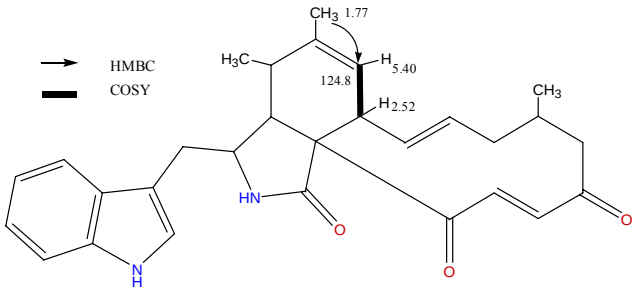


Figure 5.31: Planar structure of compound F7301-2

The relative configuration of F7301-2 (**Figure 5.32**) was assigned by comparison of the coupling constants for compound F7301-1 with those for cytochalasin Z1 (Evidente *et al*. 2002) and chaetoglobosin T (Jiao *et al.* 2004) (**Figure 5.33**). A comparison of the experimental ^{1}H NMR data of F7301-1 and 2 with the literature data is shown in **Table 5.9**, with the data displayed in red representing the chemical shift values used to assign the relative configuration.

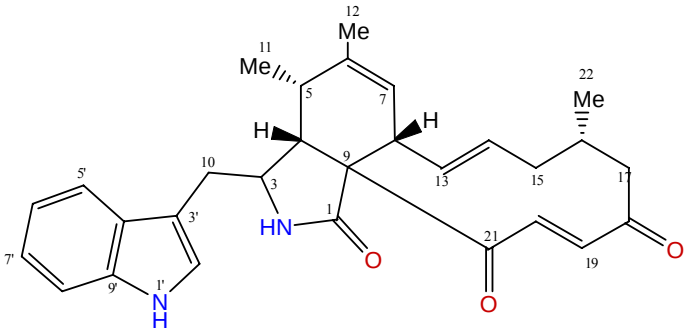
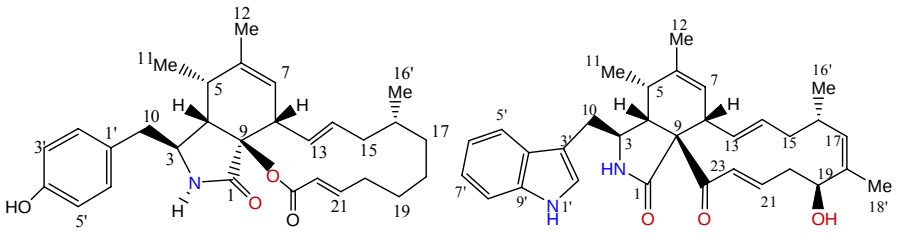


Figure 5.32: Structure of F7301-2



a) Cytochalasin Z1 b) Chaetoglobosin T

Figure 5.33: Cytochalasin Z1 and Chaetoglobosin T.

To date, more than 50 cytochalasins have been isolated from a range of fungi. Their diverse biological activity especially the cytoplasmic cleavage inhibition in mammalian cells was unprecedented in any other class of chemical compounds (Natori and Yahara, 1991). The two new cytochalasins (F7301-1 and F 7301-2) isolated in this study showed moderate cytotoxicity against murine P388 leukemia cells.

Table 5.9: ^{1}H NMR data of F7301-2, F7301-1, Cytochalasin Z1 and Chaetoglobosin T (500 MHz in $CDCl_3$)

Position	F7301-2					F7301-1					Cytochalasin Z1					Chaetoglobosin T			
	δH	splitting	*J* Hz			δH	splitting	*J* Hz			δH	splitting	*J* Hz			δH	splitting	*J* Hz	
1	-					-					-								
2	5.58	s				5.91	br s				5.53	br s				5.67	br s		
3	3.34	q	7.1	4.8	11.8	3.84	m				3.06	ddd				3.30	m		
4	3.51	t	4.5			3.34	dd	6.6	12.3		2.70	dd	4.0	3.9		2.99	t	5.0	4.0
5	2.35	d	2.4			1.73	m				2.81	m				2.50	dd	7.5	
6	-					-					-								
7	5.40	d				2.87	d	5.5			5.38	br s				5.34	br s		
8	2.52	d	10.5			2.18	m				3.20	br d	10.2			2.67	m		
9											-								
10a	3.15	dd	3.1	14.4		2.98	dd	3.3	13.8		2.92	ddd	13.4	3.6		3.03	dd	14.0	10.5
10b	2.66	q	10.5	3.7	14.3	2.73	dd	9.2	14.3		2.81	dd	13.4	10.4		2.58	dd	13.5	10.0
11	1.39	d	7.3			1.15	d	7.5			1.22	d	6.6			1.34	d	7.5	
12	1.77	s				1.27	s				1.77	br s				1.75	s		
13	6.08	m				6.26	m				5.87	ddd	14.4	10.2	1.8	5.96	m		
14	5.09	m	3.3	11.5	14.8	5.18	ddd	3.3	11.0	15.2	5.35	ddd	14.4	10.9	3.7	5.10	m		
15a	2.32	d	2.4			2.34	d	14.1			2.12	br d	13.8	1.8		2.21	m		
15b	2.13	d				2.16	m				177	m				1.90	m		
16	1.81	m				1.64	m				1.41	m				2.47	br m		
16' †											0.87	d	6.6			0.95	d	7.0	
17a	3.34	q	7.1	4.8	11.8	3.47	br s				1.60	m				5.22	d	8.0	
17b	2.11	m				2.14	m				0.93	m							
18	-					-					1.19	m							
18'											0.78	m				1.52	d	1.0	
19	8.18	d	16.5			7.96	d	16.5			1.73	m				4.38	m		
19'											1.35	m							
20	6.29	d	16.7			6.30	d	16.3			2.28	m				2.48	m		
21	-					-					7.17	ddd	15.6	9.0	6.5	6.80	m		
22	1.16	d	6.9			1.18	d	7.0			5.69	d	15.6			6.89	d	16.0	
23																			
1'																8.22	br s		
2'	7.03	s				7.07	s				7.05	d	8.4			6.99	d	2.5	
3'	-					-					6.77	d	8.4						
4'	-					-													
5'	7.39	d	8.1			7.55	d	7.89			6.77	d	8.4			7.51	d	7.5	
6'	7.22	t	7.3			7.17	t	7.52			7.05	d	8.4			7.12	t	7.5	
7'	7.16	t	7.2			7.23	t	7.3								7.19	t	7.5	
8'	7.54	d	8.0			7.40	d	8.26								7.36	d	75	
9'	-					-													

† H-16′ in cytochalasin Z1 and chaetoglobosin T is equivalent to H-22 in F7301-2.

Part B: *Chaetomium globosum*

5.7 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 654_4@20.34, identified as belonging to the genus *Chaetomium*, originated from soil obtained from Nelson and was isolated from a 4% phenol plate. Colonies on MYPA reached 85 mm in diameter in 10 days at 20 °C and showed a pale yellow aerial mycelium; **Figure 5.34a**. Colonies on SDA reached 48 mm in diameter after 10 days at 20 °C; **Figure 5.34b**, with buff aerial mycelium with a yellow exudate.

Perithecia matured within 10-12 days, olivaceous, spherical or ovate, ostiolate, 75-145 × 173-205 µm (**Figure 5.34c**); ascomatal hairs were numerous, wavy or very loosely coiled and intertwined at tips. Evanescent asci containing 8 ascospores (**Figure 5.34d**); ascospore ovate 6-7 × 8-9 µm, with an apical germ pore (**Figure 5.34e** and **f**).

A comparison of the colony and morphology features with that of the description provided by Arx *et al.* (1984, 1986) and Guarro and Figueras (1989) enabled strain 654_4@20.34 to be identified as *Chaetomium globosum* Kunze.

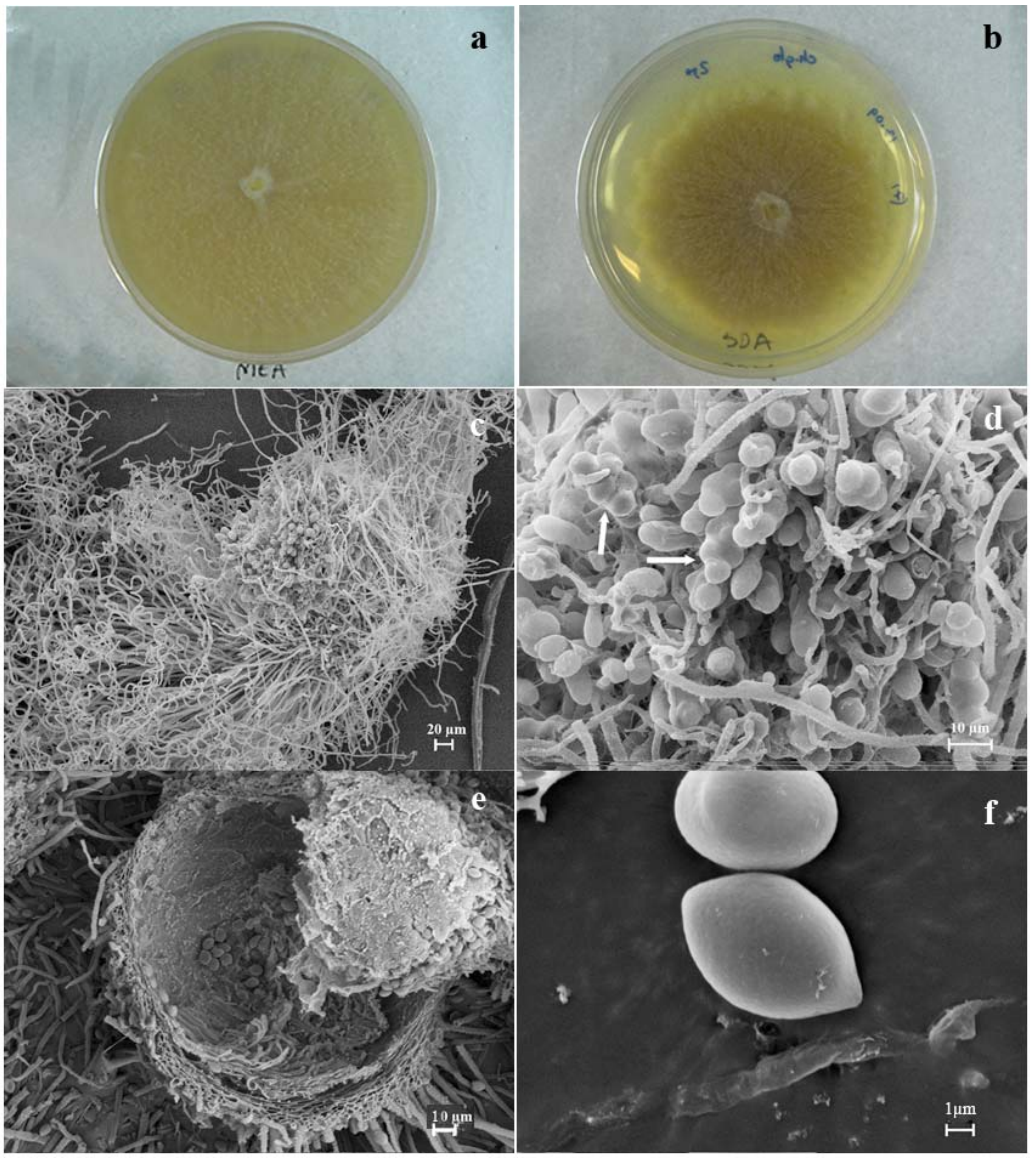


Figure 5.34: *Chaetomium globosum*; **a)** 10 days colony on MYPA plate; **b)** 10 days colony on SDA plate; **c)** SEM of ascocarp (bar = 20 µm); **d)** SEM of asci with an immature ascospores (bar =10 µm); **e)** SEM of cross secion of perithecium (bar = 1 µm); **f)** SEM of ascospores (bar = 1 µm).

5.8 EFFECT OF ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.8.1 Effect of elicitors on growth of *C. globosum*

C. globosum was grown on MYPA and SDA media at 20 °C for 20 days. The colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of mycelial growth of *C. globosum* in the presence of elicitors in MYPA and SDA media is shown in **Figures 5.35** and **5.36** respectively. Ascocarp production was observed, in the presence of some elicitors and is presented in **Table 5.10**

Table 5.10: Observation of ascomata production at 20 days incubation.

Elicitors	Observation of visible ascocarps	
	MYPA	SDA
Control	++	++
TTC	+	+
MPA	+++	++
TCZ	+	+
NYS 100 U	++	+
NYS 10 U	+	+
NYS 1 U	+	++
CLY 10 µM	+	+
CLY 5 µM	+++	+
CLY 2.5 µM	++	+
PHA 0.1 U	+++	++
LB 0.5 µg	N/O	N/O
LB 0.25 µg	+	++
JAS 0.05 µM	+	++
JAS 0.01 µM	++	++
HDACI IV 1 µM	N/O	N/O
HDACI IV 0.5 µM	+	N/O
HDACI IV 0.05 µM	+	+
HDACI II 0.8 µM	N/O	N/O
HDACI II 0.4 µM	N/O	N/O
HDACI II 0.2µM	N/O	N/O

* +++ visible ascomata covering entire colony, ++ visible ascomata abundance at the edge of the colony, + visible ascomata under dissection microscope, N/O no ascomata production.

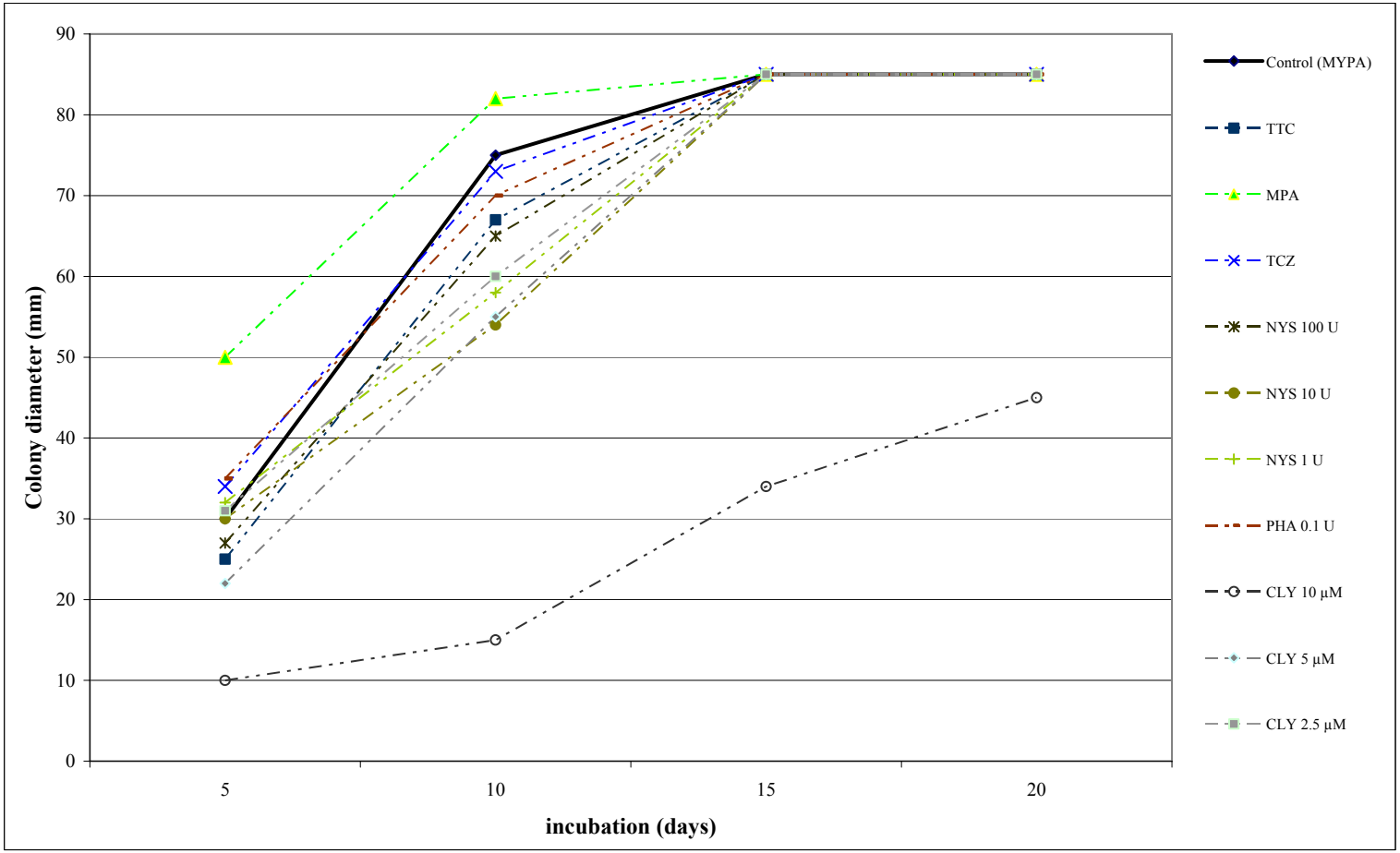


Figure 5.35: Effect of elicitors on growth of *C.globosum* on MYPA medium at 20 °C.

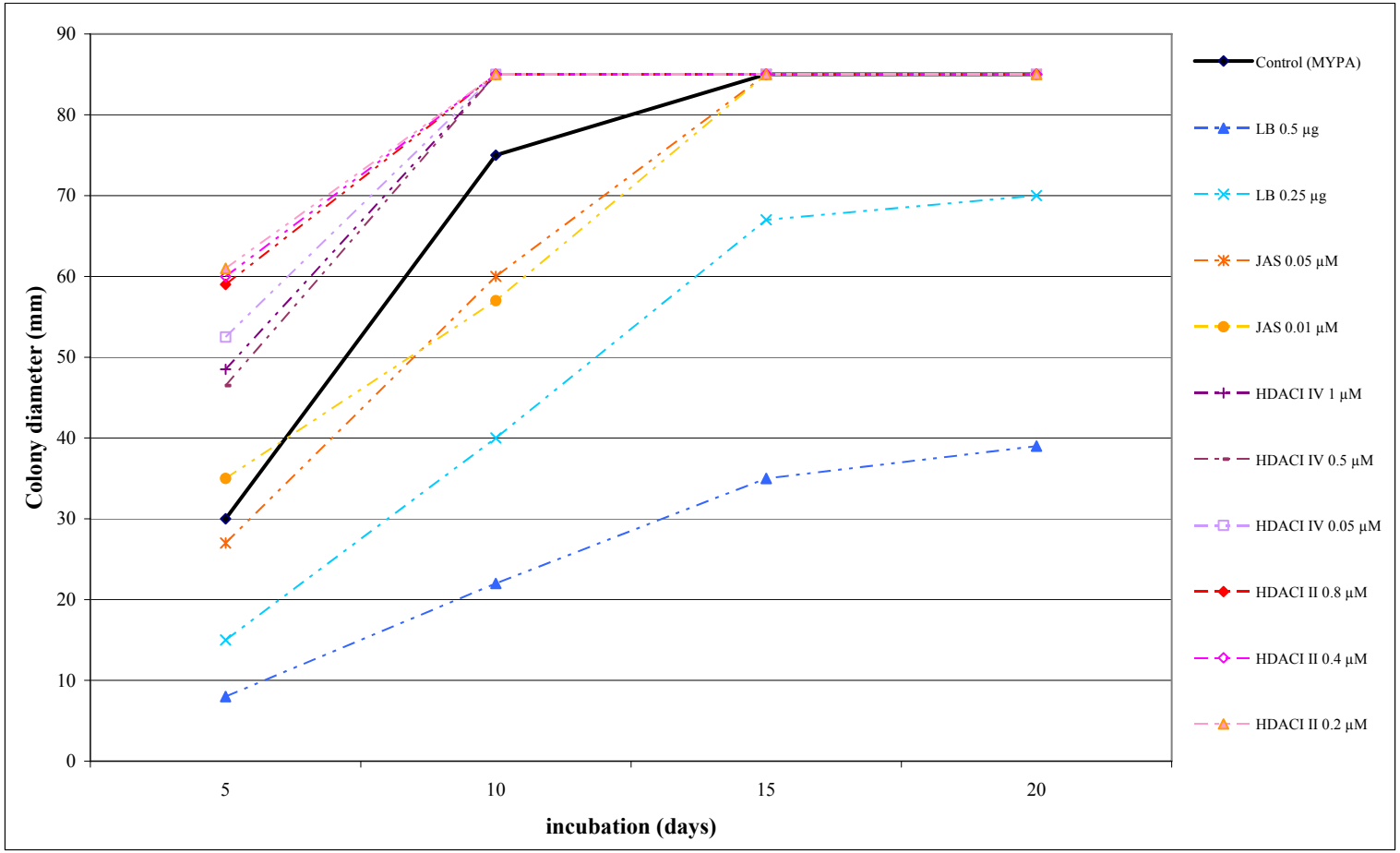


Figure 5.35: (Continued)

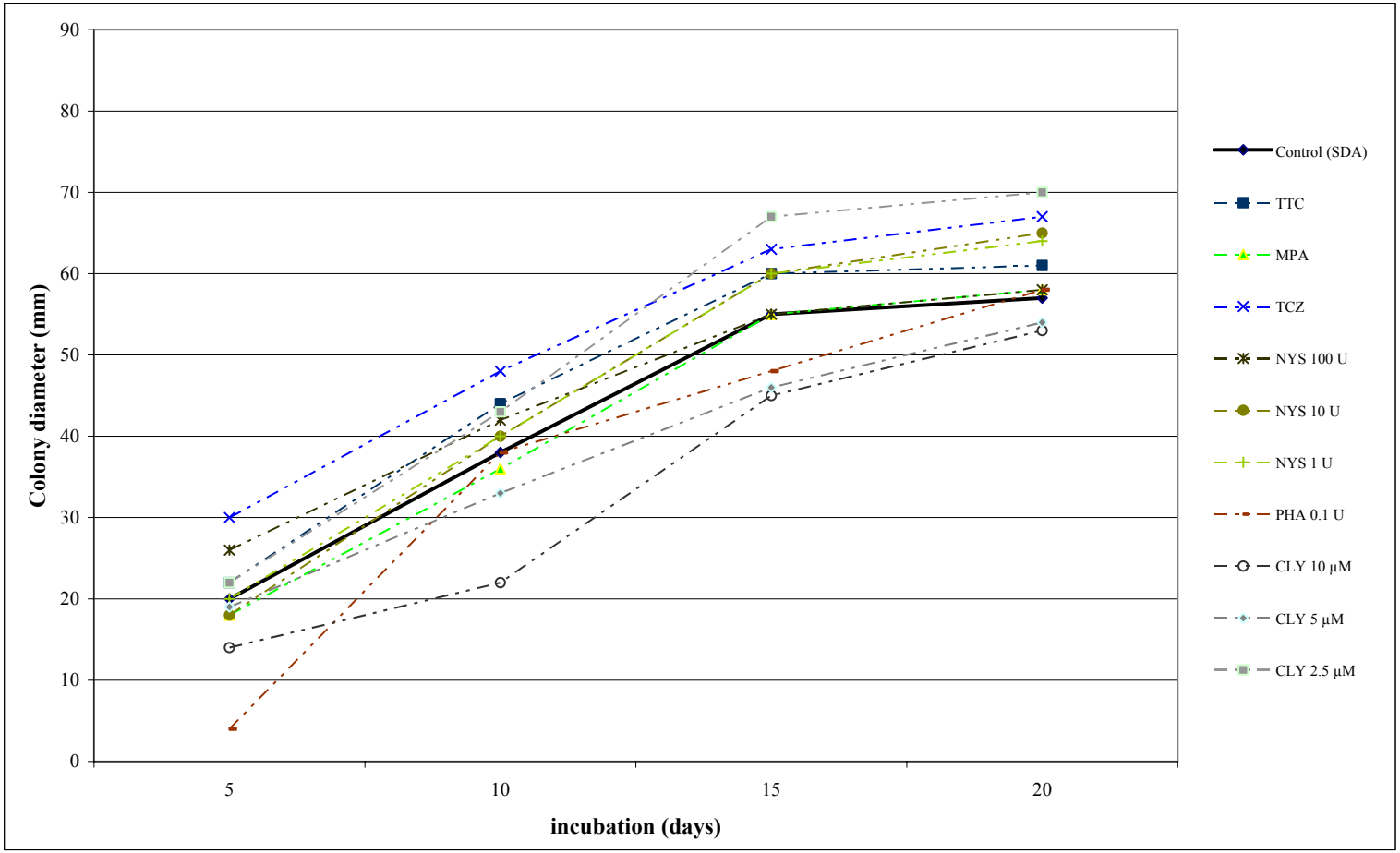


Figure 5.36: Effect of elicitors on growth of *C.globosum* on SDA medium at 20 °C.

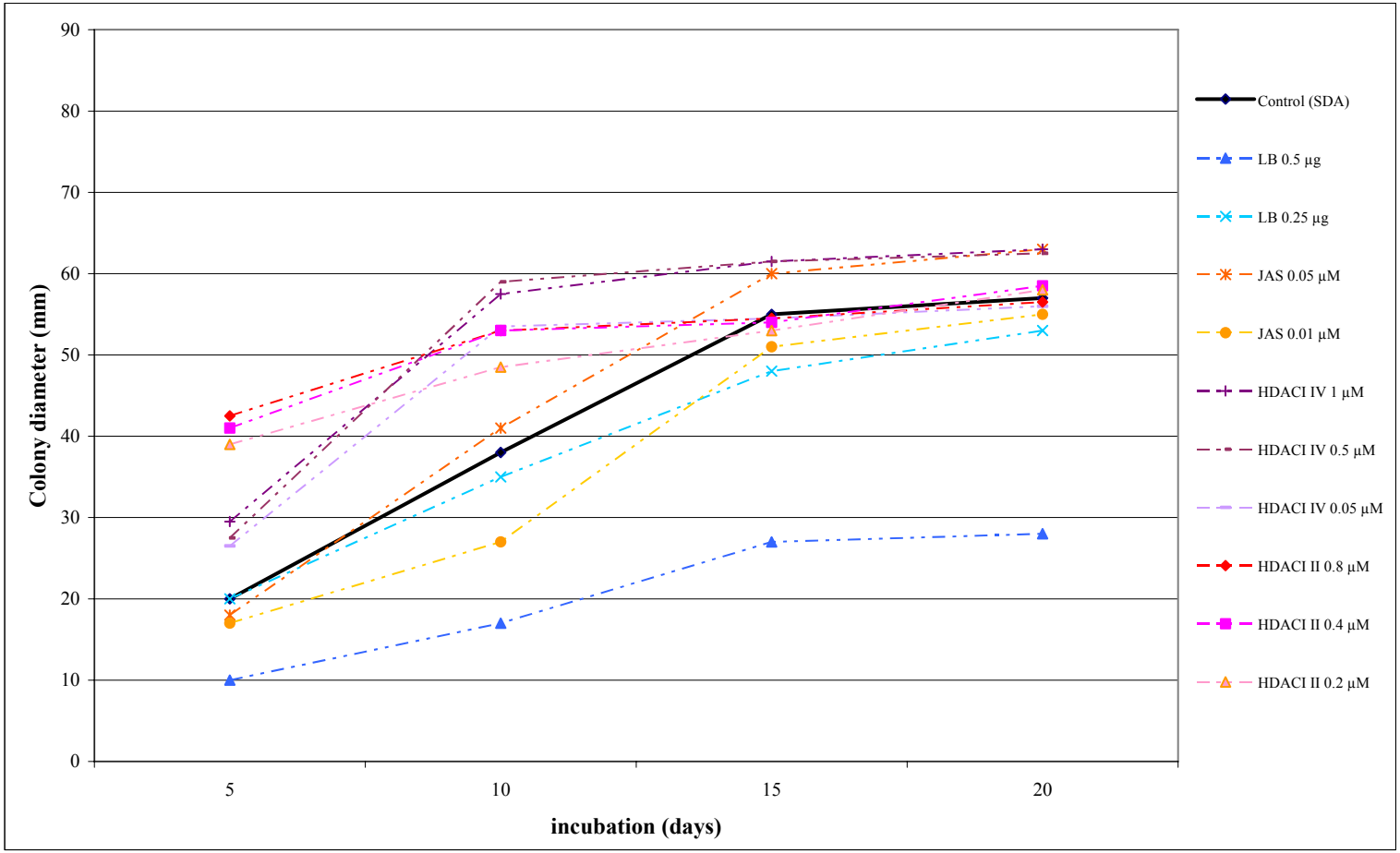


Figure 5.36: (Continued)

The growth of *C. globosum* was greater on MYPA medium than on SDA medium. On MYPA medium, colonies reached the edge of an agar plate (85 mm) after 10-15 days of incubation while on SDA medium, growth of the fungus reached stationary phase after 10-15 days of incubation. Growth of the fungus on both media was not affected by elicitors, except for latrunculin B (LB) 0.25 µg and cycloheximide (CLY) 10 µM on MYPA medium and LB 0.5 µg in both media. Ascomata development was affected when HDACI II, HDACI IV or LB 0.5 µg was present in the media, but they did not affect mycelial growth.

5.8.2 Effect of elicitors on cytotoxic and metabolite production

5.8.2.1 Cytotoxicity

There were no differences in IC_{50} values when this fungus was grown on MYPA or SDA media. Extracts from each elicitor treatment showed good activity against P388 cells except extract F9246 (yield from fungus grown on MYPA medium in the presence of nystatin (NYS) 10U) and extract F9536 (yield from fungus grown on SDA medium in the presence of HDACI II 0.8 µM) see **Table 5.11** for details.

Table 5.11: Effect of elicitors on cytotoxicity of *C.globosum* on MYPA and SDA media culture at 20 °C for 20 days.

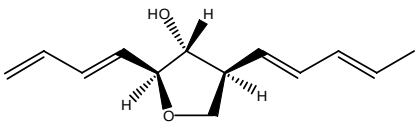
Elicitors	Cytotoxicity			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9240	2,775	F9255	2,667
TTC	F9241	3,020	F9256	2,996
MPA	F9242	1,484	F9257	3,020
TCZ	F9243	6,971	F9258	2,579
NYS 100 U	F9244	6,971	F9259	1,484
NYS 10 U	F9245	6,715	F9260	3,020
NYS 1 U	F9246	>12,500	F9261	7,100
CLY 10 µM	F9247	2,290	F9262	2,579
CLY 5 µM	F9248	2,157	F9263	5,431
CLY 2.5 µM	F9249	5,559	F9264	2,994
PHA 0.1U	F9250	6,971	F9265	3,020
LB 0.5 µg	F9251	8,431	F9266	9,793
LB 0.25 µg	F9252	3,232	F9267	8,687
JAS 0.05 µM	F9253	4,916	F9268	5,542
JAS 0.01 µM	F9254	3,330	F9269	7,290
HDACI IV 1 µM	F9435	9,504	F9442	8,687
HDACI IV 0.5 µM	F9437	9,054	F9444	9,054
HDACI IV 005 µM	F9439	8,687	F9446	6,971
HDACI II 0.8 µM	F9530	8,182	F9536	>12,500
HDACI II 0.4 µM	F9532	6,971	F9538	7,100
HDACI II 0.2 µM	F9534	6,063	F9540	6,971

5.8.2.2 HPLC screening

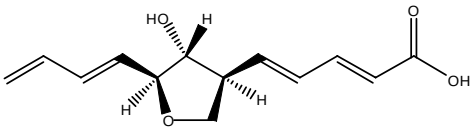
A comparison of ELSD traces (**Figure 5.38 and 5.39**) showed that *C. globosum* produced different metabolites when grown on different media. Without elicitors, tetrahydrofuran B (**Figure 5.37a**) was the main compound produced when grown on MYPA, while aureonitol (tetrahydrofuran A) (**Figure 5.37b**) was the main compound produced when the fungus was

grown on SDA. The HPLC traces in Figures **5.38** and **5.39** revealed that in the presence of certain elicitors, as listed in **Table 5.12**, tetrahydrofuran A and B were produced together on both media. An inducement of peak F9242-2, which was later identified as chaetoviridin A (**Figure 5.37c**), occurred in the MYPA extracts when certain elicitors (as listed in **Table 5.12**) were added to the medium.

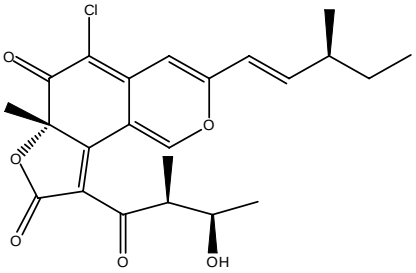
A production of peak F9245-2 (later identified as chaetoglobosin A (**Figure: 5.37d**) by *C. globosum* was also induced when some elicitors as listed in **Table 5.12** were added to both media. Structural elucidation of these four compounds is given in **section 5.9**.



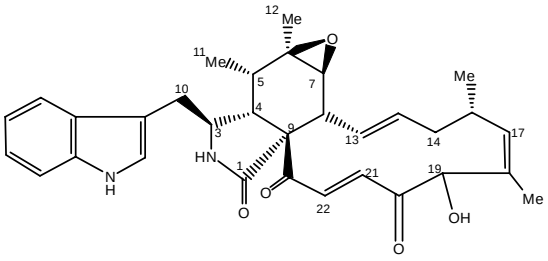
a) Tetrahydrofuran A



b) Tetrahydrofuran B



c) Chaetoviridin A



d) Chaetoglobosin A

Figure 5.37: Compounds produced by *C. globosum*

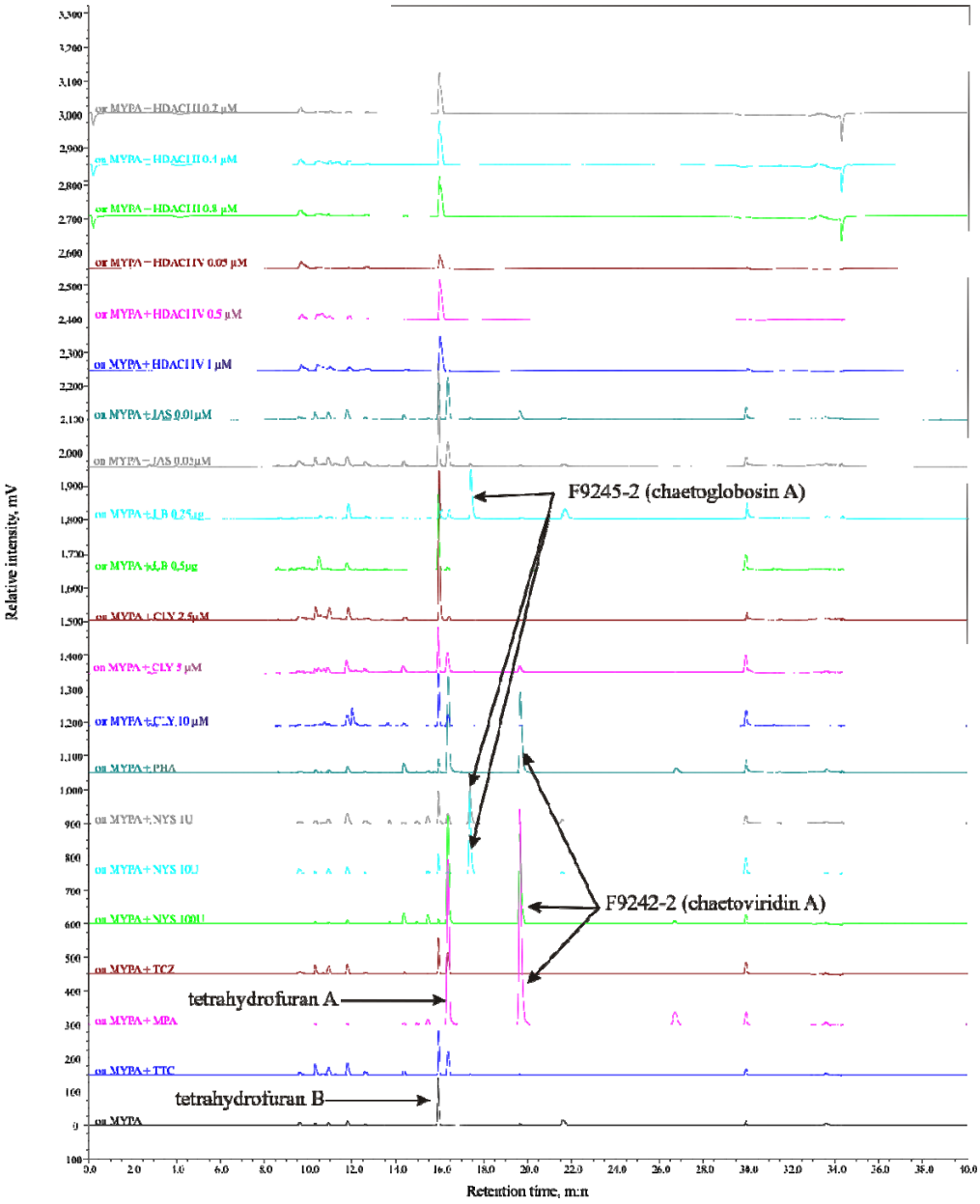


Figure 5.38: HPLC traces of extracts from *C. globosum* on MYPA medium at 20°C for 20 days with addition of different elicitors.

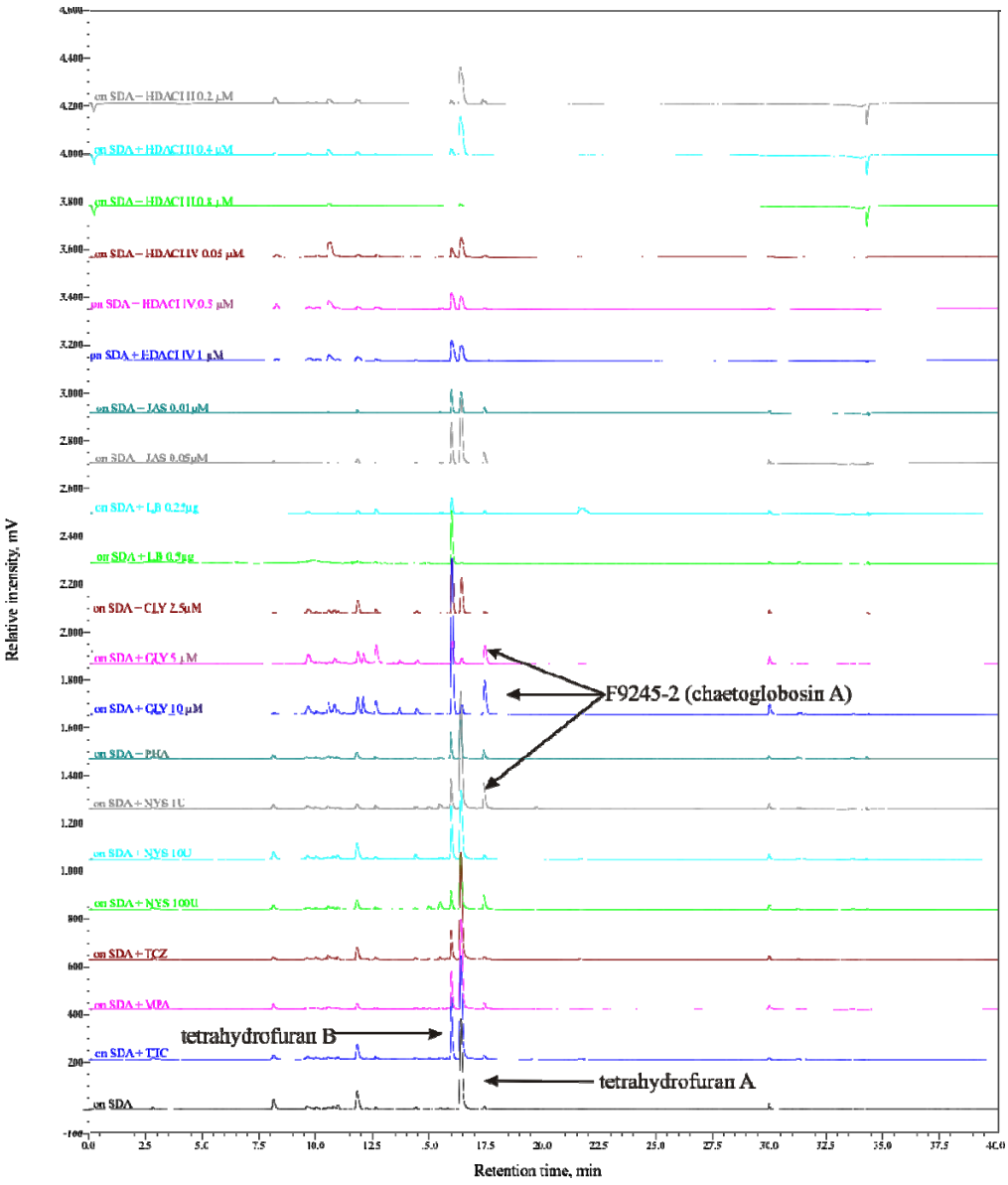


Figure 5.39: HPLC traces of extracts from *C. globosum* on SDA medium at 20°C for 20 days with addition of different elicitors.

Table 5.12: Summary of metabolite peaks enhanced by elicitors

Summary of metabolite peaks					
Metabolites	**Peak name**	**Elution (min)**	**Media**	**Elicitors**	**Note**
Tetrahydrofuran A	F7392-E8	16.34	SDA	none	Original peak
			MYPA	CLY 5 µM JAS 0.05 µM JAS 0.01 µM MPA NYS 100 U PHA 0.1 U	Induced peak
Tetrahydrofuran B	F9240-1	15.94	MYPA	None	Original peak
			SDA	CLY 10, 5, 2.5 µM JAS 0.05, 0.01 µM LB 0.5 µg MPA NYS 100, 10, 1 U PHA 0.1 U TTC TCZ	Induced peak
Chaetoviridin A	F9242-2	19.64	MYPA	MPA NYS 100 U PHA 0.1 U	Induced peak
Chaetoglobosin A	F9245-2	17.36	MYPA	NYS 10 U NYS 1 U LB 0.25 µg	Induced peak
			SDA	CLY 10 µM CLY 5 µM	Induced peak

5.9 STRUCTURAL ELUCIDATION OF COMPOUNDS PRODUCED BY *C. globosum*

5.9.1 Peak F7392-E8

The crude extract (F7392) from SDA; 20 °C for 30 days was screened for bioactivity and showed potent activity (>80% cells inhibition) in both the P388 and *B. subtilis* quick screens, with subsequent determination of the P388 IC_{50} value of 2,667 ng/mL. The HPLC profile (**Figure 5.40**) showed a distinct peak, F7392-E8, which eluted over the bioactive region at 16.34 minutes.

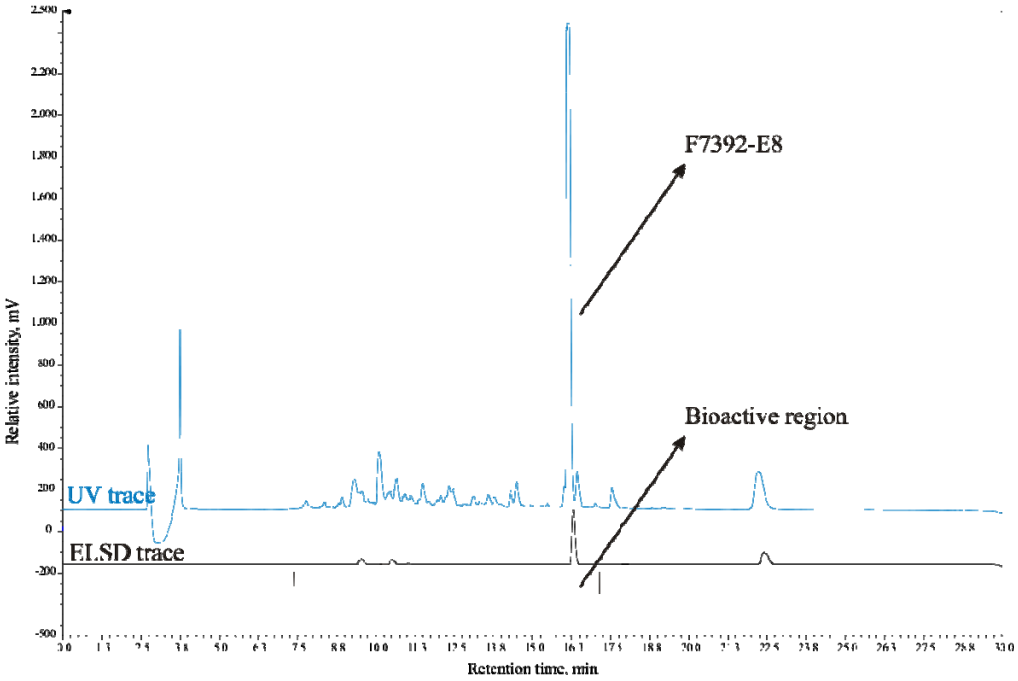


Figure 5.40: HPLC chromatogram of extract F7392 showing overlay of ELSD detection (black) and the UV detection (blue) of peak F7392-E8.

Results of HPLC microtitre screening showed that peak F7392-E8 was active against P388 cells, but inactive against *B. subtilis*. The extracted UV spectrum

of F7392-E8 (**Figure 5.40**) showed no exact match to any compounds in the HPLC-UV/R_t library database. ESIMS of compound F7392-E8 suggested the molecular weight to be 415 amu ([M+H^+]) **(Figure 5.42).** Peak F7392-E8 was further investigated using the CapNMR technique.

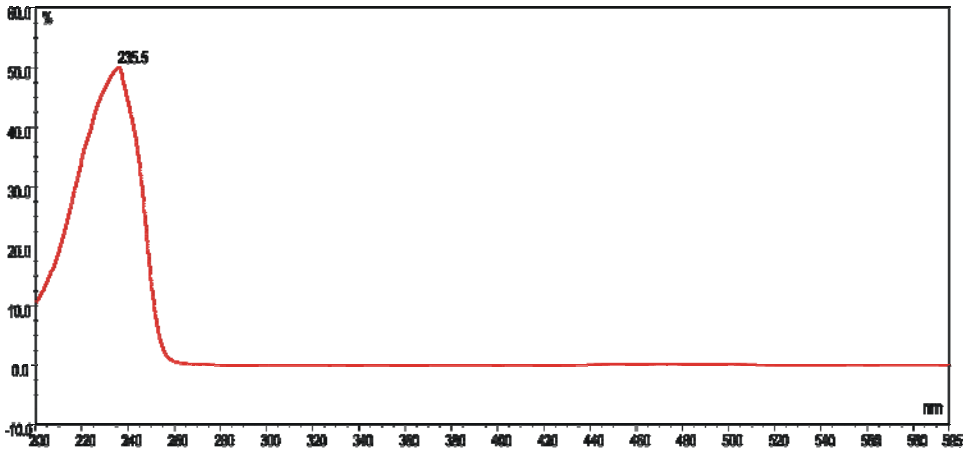


Figure 5.41: UV absorption (nm) in % of UV-max of compound F7392-E8

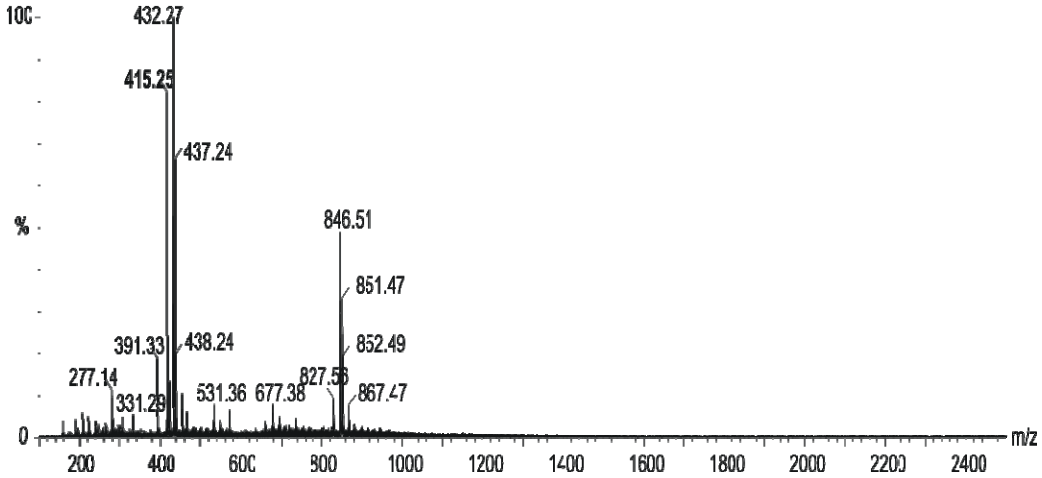


Figure 5.42: ESIMS spectrum of compound F7392-E8

The ^{1}H NMR spectrum of F7392-E8 (**Figure 5.43**) revealed the presence of one doublet methyl group at δ_H 1.72. The signals at δ_H 5.09, 5.21, 5.46, 5.64, 5.72, 6.03, 6.12, 6.29 and 6.35 all represented olefinic protons in the compound. The

integral in the ^{1}H NMR spectrum ibdicated that the resonances at δ_H 3.68 and 4.05 were each two protons.

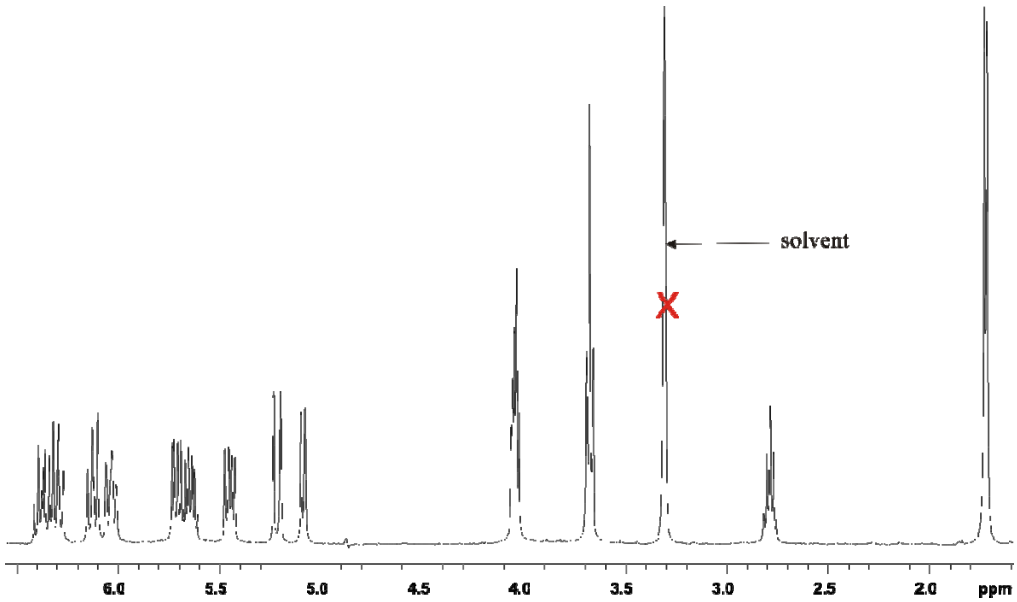


Figure 5.43: ^{1}H NMR spectrum of F7392-E8; 44 µg in 6 µL of CD_3OD, recorded 500 MHz, 64 sec.

From the COSY NMR experiment (**Figure 5.44**) two different fragments were deduced.

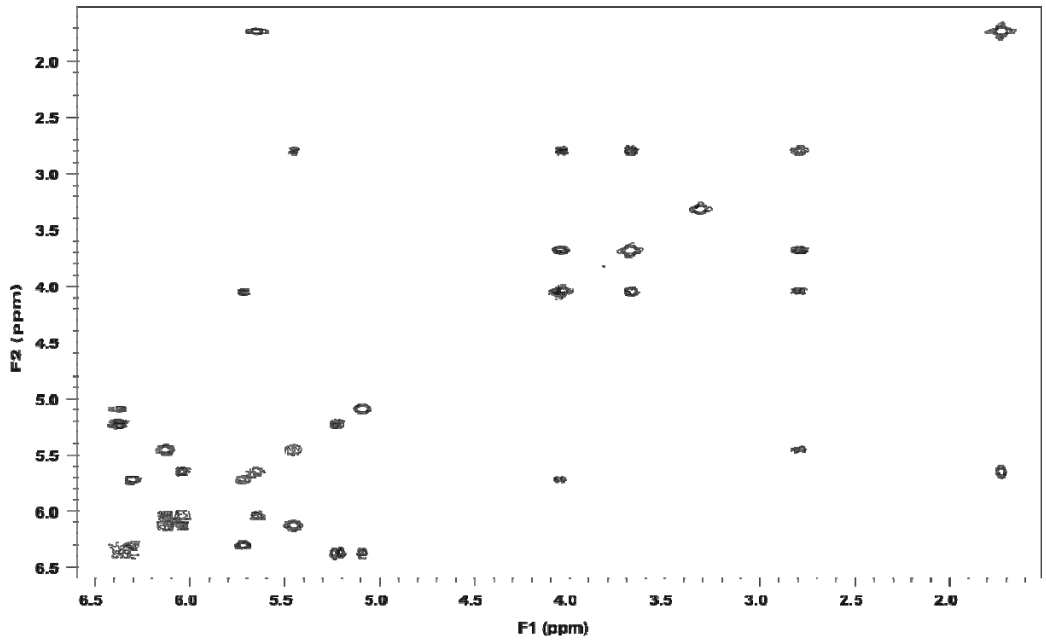


Figure 5.44: COSY spectrum of F7392-E8 in CD_3OD.

The first fragment was assembled from units revealed in the COSY experiment, showing that the methyl protons at δ_H 1.72 were coupled to an olefinic proton at δ_H 5.64, which was in turn coupled to an olefinic proton at δ_H 6.03. Following through the correlation in the COSY experiment it was possible to trace a sequence of coupled protons from the methyl doublet at δ_H 1.72 to 5.64, 6.03, 6.12, 5.46, 2.79 to the methylene at δ_H 3.68/4.05 (see **Figure 5.45**).

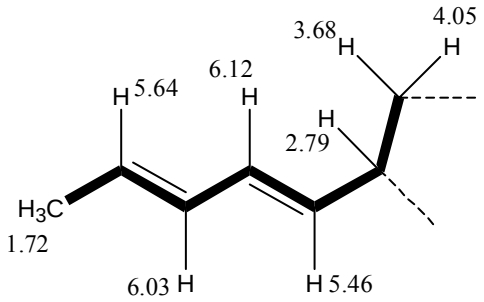


Figure 5.45: Spin system in fragment 1 of F7392-E8 obtained by COSY (solid line).

A second fragment was also deduced from units defined in the COSY experiment. The proton resonance at δ_H 4.05, part of the first system, was coupled to protons at δ_H 3.68 and 5.72, while the olefinic proton at δ_H 5.72 was coupled to the δ_H 6.29 proton, which was in turn was coupled to δ_H 6.35, and finally to a methylene group at δ_H 5.09 and 5.21 (**Figure 5.46**).

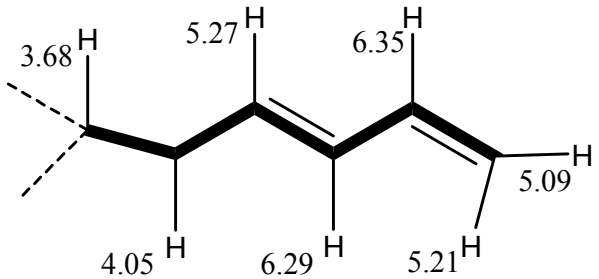


Figure 5.46: Spin system in fragment 2 of F7392-E8 obtained by COSY (solid line).

A search of the AntiMarin database using these two substructures and restricting the search to the presence of one doublet methyl group resulted in only one hit. This was aureonitol (Tetrahydrofuran A) (mw: 206.28) (**Figure 5.47**) which had been derived from *Chaetomium cochiliodes* (Abraham and Arfman, 1992).

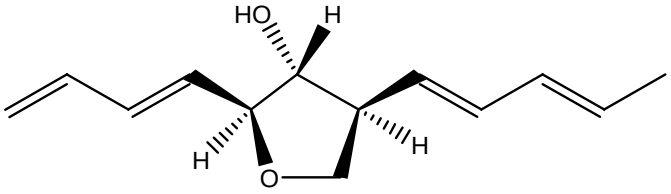


Figure 5.47: Aureonitol

The ^{1}H NMR spectrum of F7392-E8 was reacquired in $CDCl_3$ in order to direclty compare the experimental NMR data with the literature data. The details are summarised in **Table 5.13**.

Table 5.13: ^{1}H NMR data for aureonitol[†] and F7392-E8[‡]

	F7392-E8 in CD_3OD							F7392-E8 in $CDCl_3$				aureonitol in $CDCl_3$				
Position	**δ_H**	**splitting**	***J* Hz**		**COSY**			**δ_H**	**splitting**	***J* Hz**		**δ_H**	**splitting**	***J* Hz**		
1	1.72	d	6.3		5.64			1.74	d	6.6		1.74	dd	6.5	1.5	
2	5.64	m	6.8	14.6	1.72	6.03		5.67	dd	7.5	14.7	5.65	dq	6.5	14.5	
3	6.03	t	14.8	10.7	5.64	6.12		6.02	t	14.9	10.5	6.00	ddq	1.5	14.5	10.0
4	6.12	dd	10.3	14.9	5.46	6.03		6.12	dd	10.5	14.9	6.12	dd	10.0	15.0	
5	5.46	dd	8.4	14.9	2.79	6.12		5.44	dd	8.6	15.0	5.40	dd	15.0	9.0	
6	2.79	m	8.0	7.8	3.68	4.05	5.46	2.84	m			2.84	dddd	9.0	7.5	
7	3.68	t	8.3		2.79	4.05		3.72	m			3.72	dd	7.5		
8	4.05	m			3.68	5.72		4.13	m			4.12	dd	7.5	8.5	
9	5.72	dd	7.0	15.1	4.05	6.29		5.72	dd	7.2	14.3	5.68	dd	7.5	14.5	
10	6.29	m			5.72			6.32	m			6.32	m	10.0	14.5	
11	6.35	m			5.09	5.21		6.37	m			6.32	m	10.0	17.0	
12a	5.21	d	17.2		6.35			5.11	d	16.1		5.10	d	17.0		
12b	5.09	d	10.5		6.35			5.23	d	9.4		5.22	d	10.0		
13a	3.68	t	8.3		2.79	3.68		3.72	m			3.61	dd	8.5		
13b	4.05	m			2.79	4.05		4.13	m			4.10	dd	8.5		

[†] Specrtrum was recorded at 400 MHz for ^{1}H.
[‡] Spectra were recorded at 500 MHz for ^{1}H.

Compound F7392-E8 did not readily ionise in the mass spectrometer, therefore, the accurate molecular mass for this compound could not be obtained. Nevertheless the proton chemical shifts and coupling constants of compound F7392-E8 aureonitol as shown in **Table 5.13** were and were found to be almost identical. As such it was concluded that F7392-E8 was aureonitol.

5.9.2 Peak F9240-1

Extract F9240 was obtained from a culture on MYPA at 20 °C for 20 days. The HPLC profile (**Figure 5.48**) showed a distinct peak, F9240-1, with UV absorption maxima at 227.1 and 263.9 nm (**Figure 5.49**) which eluted at 15.99 minutes.

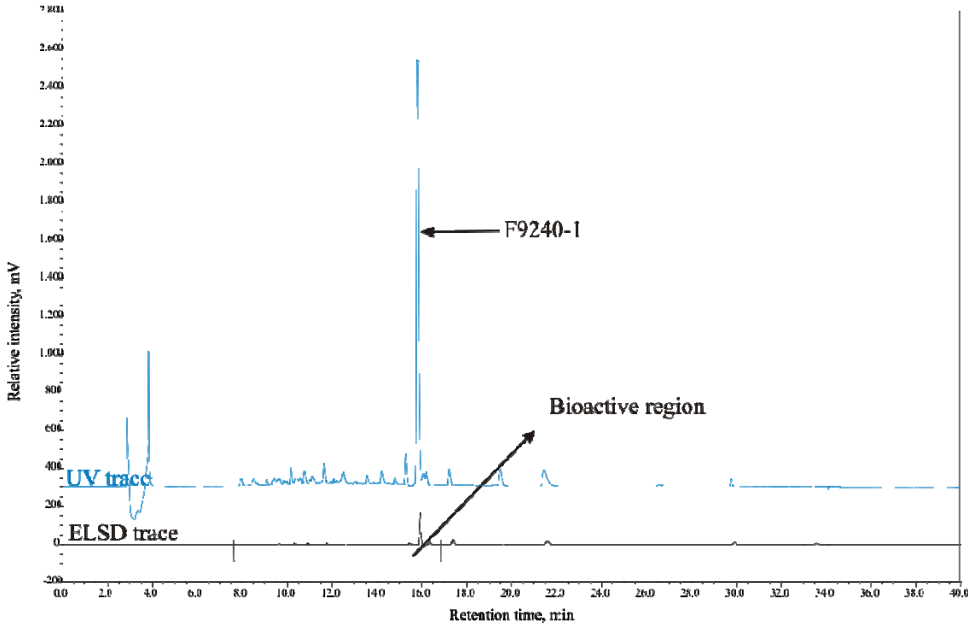


Figure 5.48: HPLC chromatogram of extract F7392 showing overlay of ELSD detection (black) and the UV detection (blue) of peak F7392-E8.

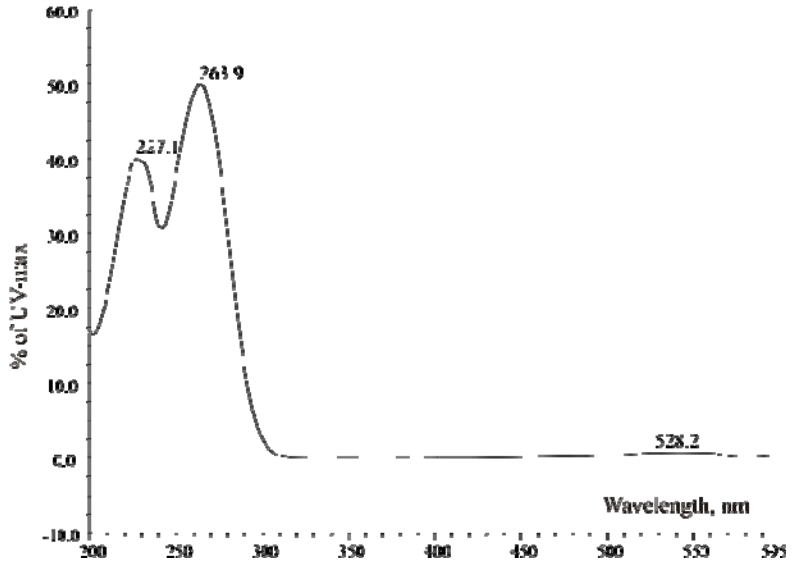


Figure 5.49: UV absorption (nm) in % of UV-max of compound F9240-1

The ^{1}H NMR spectrum of peak F9240-1 (**Figure 5.50**) showed that it was identical to that of tetrahydrofuran B (**Figure 5.51**) previously identified by Ms Francine Smith (BSc, Hons) project; University of Canterbury, 2007. Thus peak F9240-1 could be assigned as being tetrahydrofuran B.

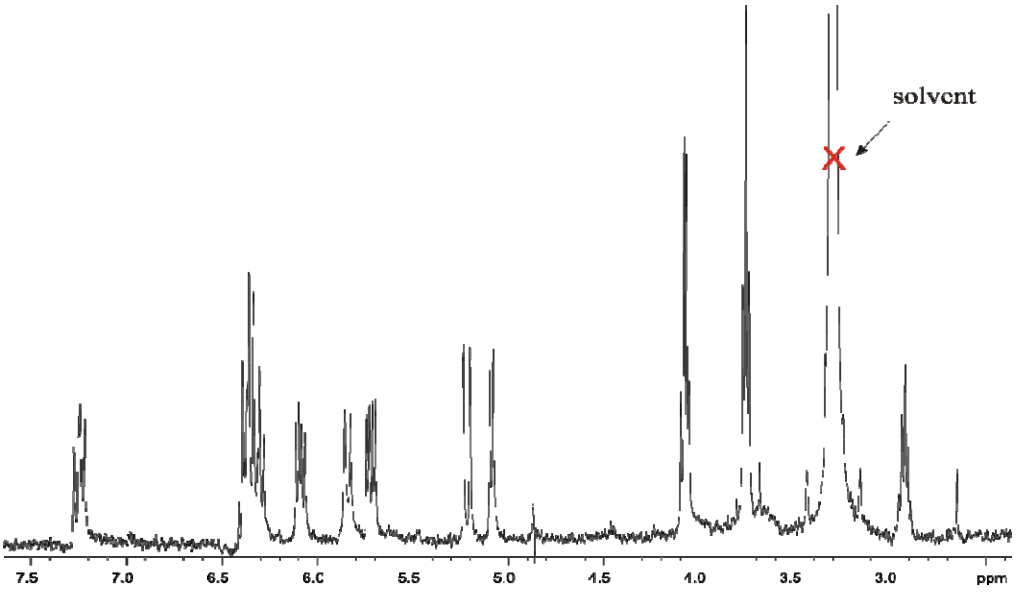


Figure 5.50: ^{1}H NMR spectrum of F9240-1; 36 µg in 6 µL of CD_3OD, recorded at 500 MHz, 240 sec.

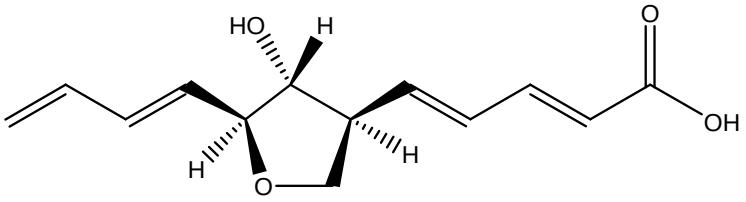


Figure 5.51: Tetrahydrofuran B

5.9.3 Peak F9242-2

Peak F9242-2 was obtained from a culture on MYPA medium containing mycophenolic acid. The HPLC profile (**Figure 5.52**) showed two distinct peaks on the ELSD trace; F9242-1 (which was identified as aureonitol) and the peak at F9242-2, which eluted at 19.6 min. The extracted UV spectrum of F9242-2 (**Figure 5.53**) showed an exact match with chaetoviridin A (R_t = 18.8 min) in the HPLC-UV/R_t library database.

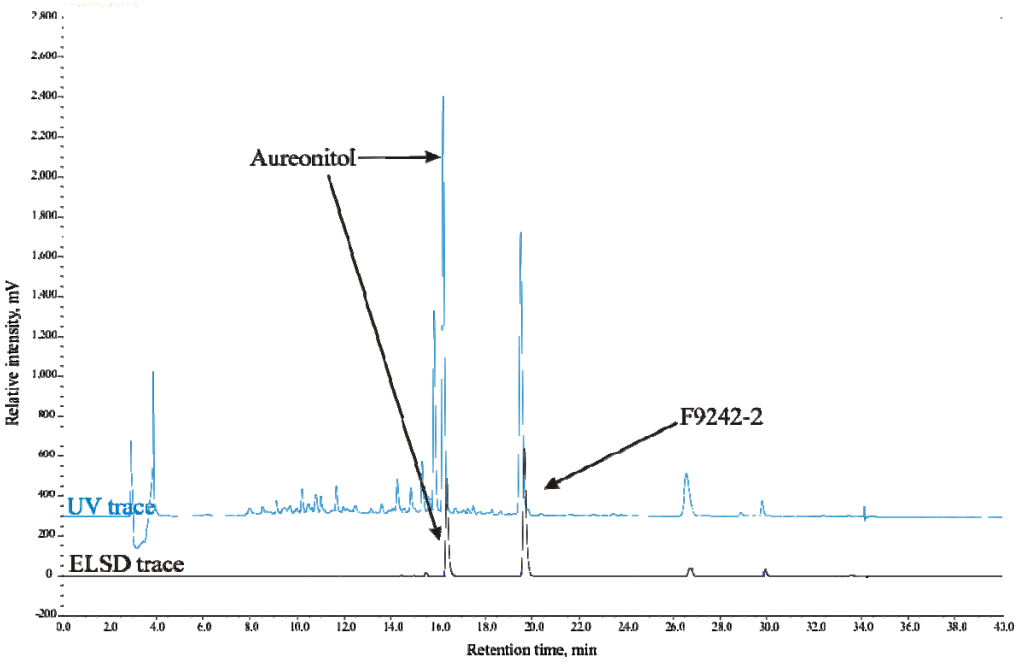


Figure 5.52: HPLC chromatogram of extract F9242 showing overlay of ELSD detection (black) and the UV detection (blue).

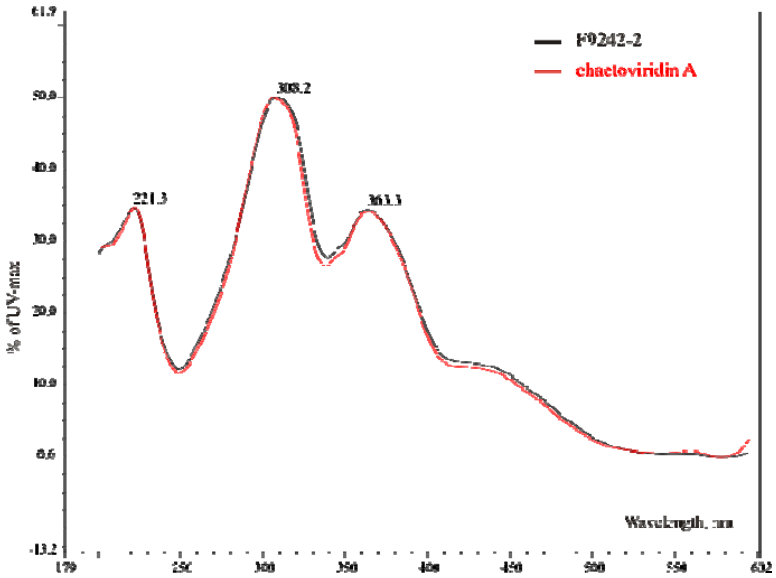


Figure 5.53: UV absorption (nm) in % of UV-max of compound F9242-2 (black) and chaetoviridin A (red)

ESIMS data for this compound (**Figure 5.54**) indicated that the mass was 433 amu ([M+H$^+$]) which is identical to the reported mass for chaetoviridin A (Takahashi *et al*. 1990).

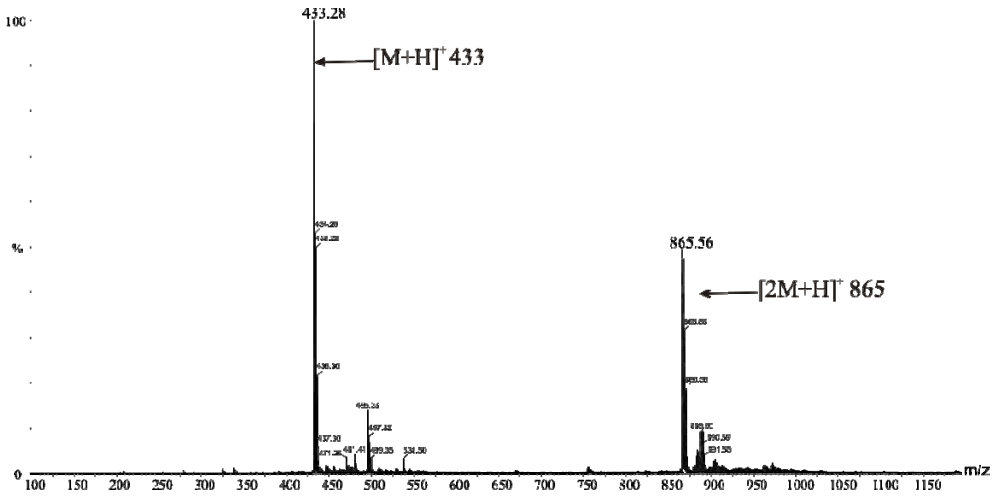


Figure 5.54: ESIMS spectrum of compound F9242-2.

The ^{1}H NMR spectrum of F9242-2 (**Figure 5.55**) clearly showed the presence of one singlet, three doublet and one triplet methyls (δ_H 0.8 – 1.8). A search of the AntiMarin database based on these five methyl groups and a molecular weight of 431-433 amu (**Figure 5.56a**) resulted in a match with chaetoviridin A (**Figure 5.56b**). A comparison of proton chemical shifts of F9242-2 with the reported data of chaetoviridin A (Takahashi *et al*. 1990) confirmed the assignment of F9242-2 as being chaetoviridin A (**Figure 5.57**).

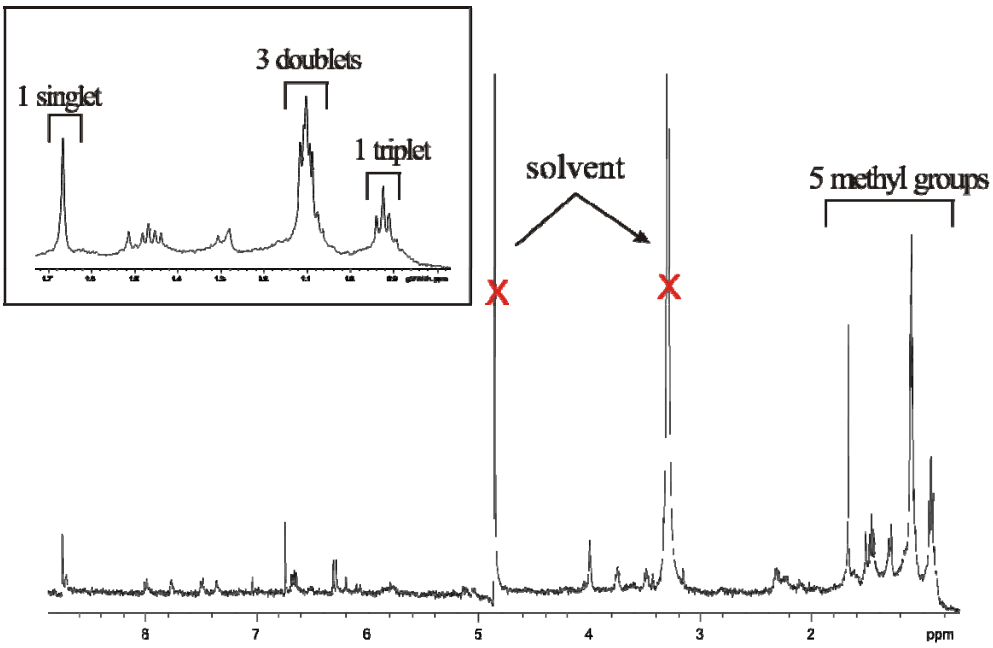


Figure 5.55: ^{1}H NMR spectrum of F9242-2; 24 µg in 6 µL of CD_3OD, recorded at 500 MHz, 240 sec. Inset: enlargement of five methyl groups.

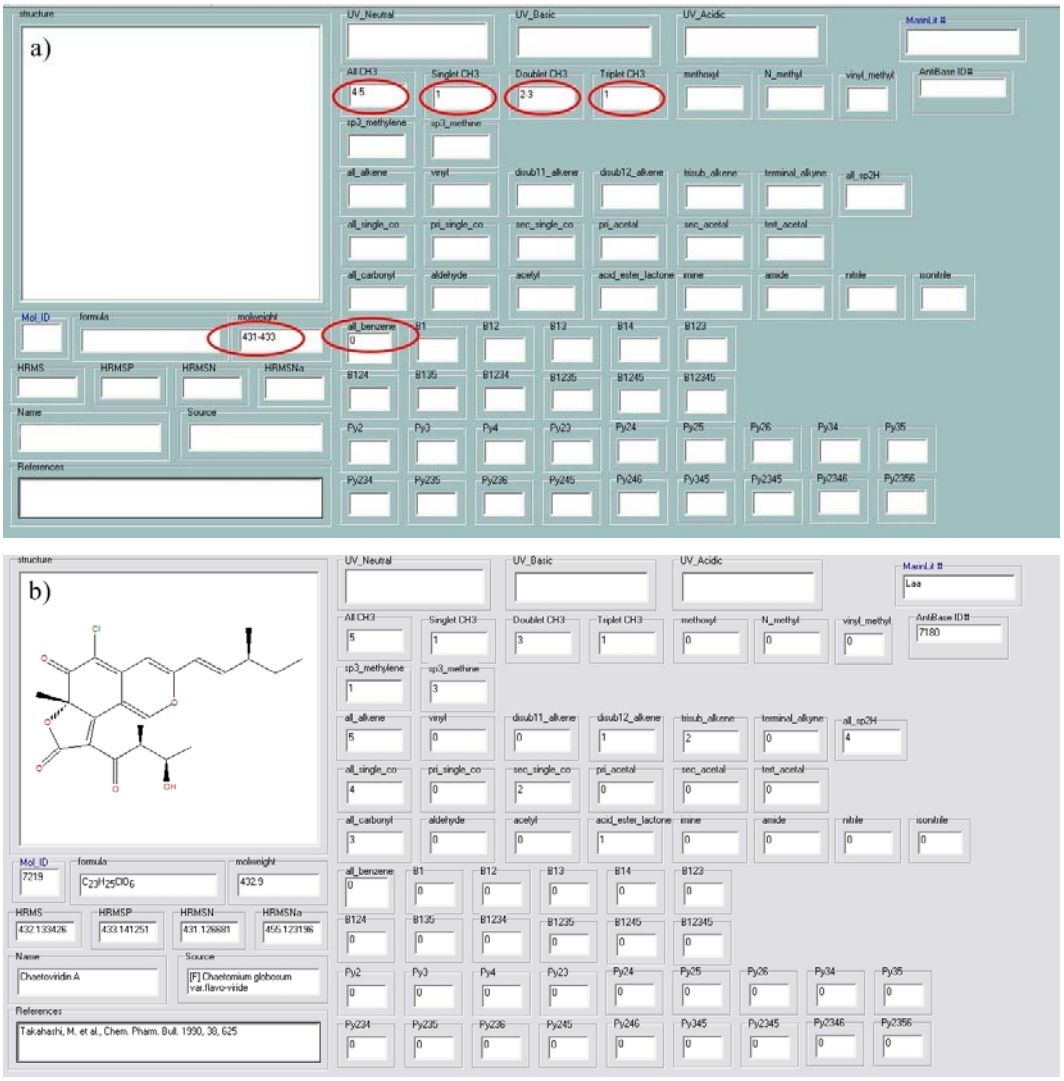


Figure 5.56: **a)** AntiMarin search profile for F9242-2; **b)**AntiMarin search result for F9242-2

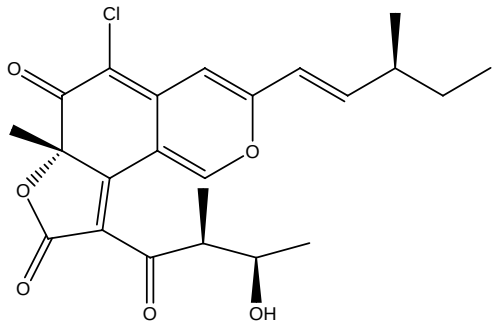


Figure 5.57: Chaetoviridin A

5.9.4 Peak F9245-2

The HPLC profile of the extract obtained from F9245 cultured on MYPA containing nystatin (10 U) revealed the presence of two major components; tetrahydrofuran B and F9245-2, which appeared at R_t 15.94 and 17.36 min respectively (**Figure 5.58**). The extracted UV spectrum of F9245-2 (**Figure 5.59**) showed no match in the HPLC-UV/R_t library database.

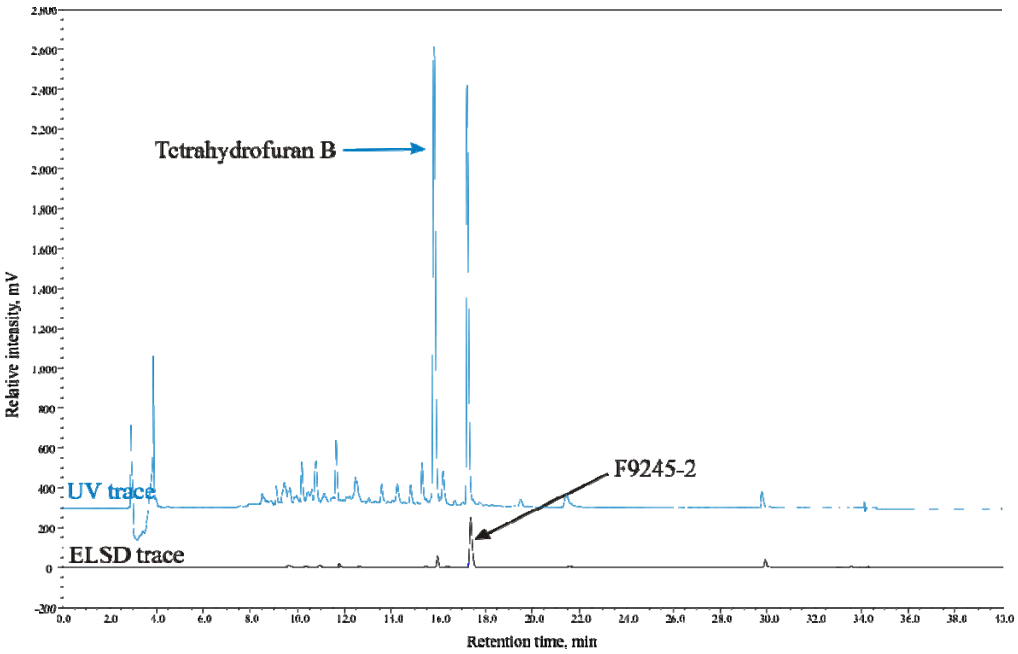


Figure 5.58: HPLC chromatogram of extract F9245 showing overlay of ELSD detection (black) and the UV detection (blue).

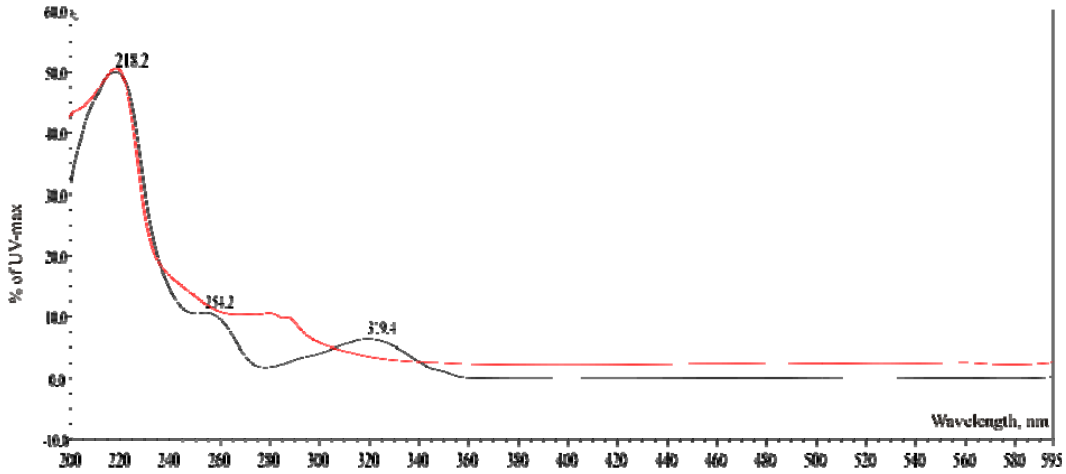


Figure 5.59: UV absorption (nm) in % of UV-max of compound F9245-2 (black) and chaetoviridin A (red)

This compound was shown to have a molecular mass of 529 amu ([M+H$^+$]) in the positive ESI MS (**Figure 5.60**). The ^{1}H NMR spectral data for compound F9245-2 clearly showed the presence of two singlet methlys at δ_H 1.27 and 1.30, two doublet methyls at δ_H 0.98 and 1.05 and an indole system at the chemical shift region δ_H 6.92-7.45 (**Figure 5.61**).

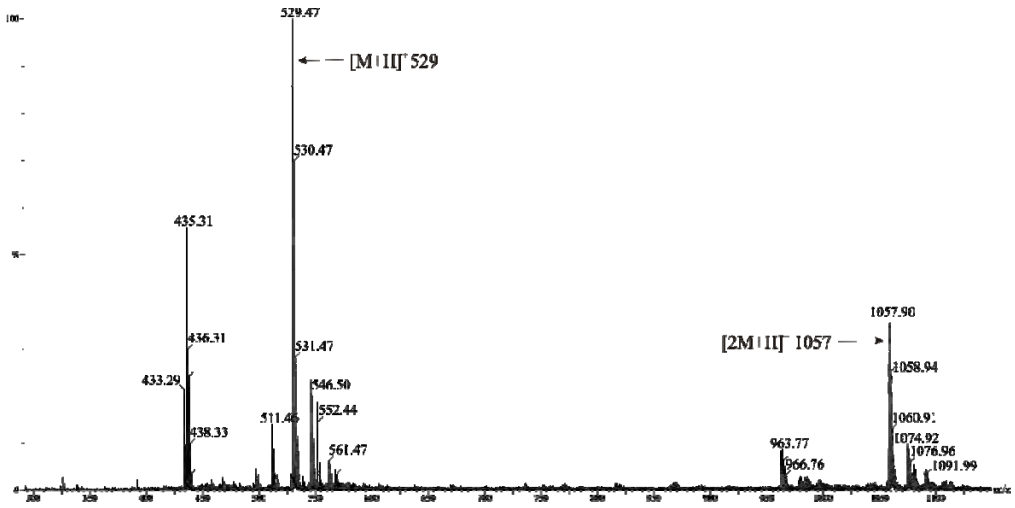


Figure 5.60: ESIMS spectrum of compound F9245-2.

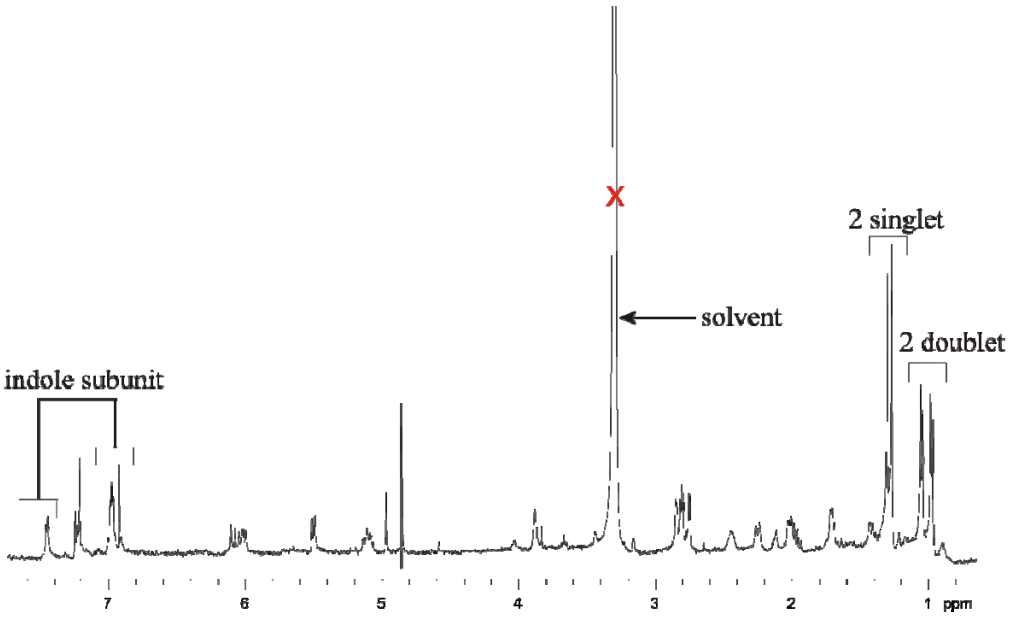


Figure 5.61: ^{1}H NMR spectrum of F9245-2; 24 µg in 6 µL of CD_3OD, recorded at 500 MHz, 360 sec.

A search of the AntiMarin database based on a total of four methyls (two singlets and two doublets), a 1,2-substituted benzene and a molecular weight of 527-529 amu (**Figure 5.62**) resulted in matches with two known compounds; chaetoglobosin A and chaetoglobosin C (**Figure 5.63**).

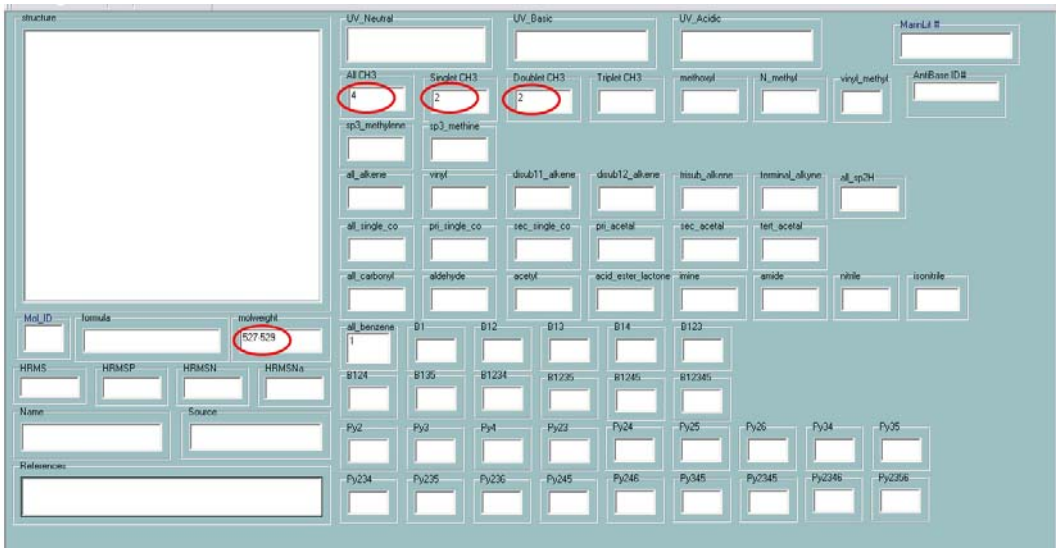
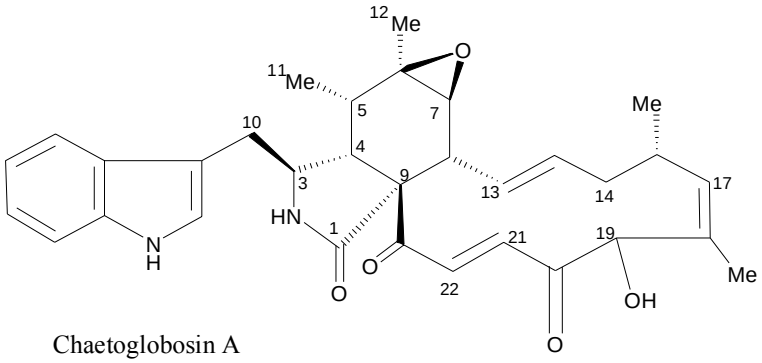
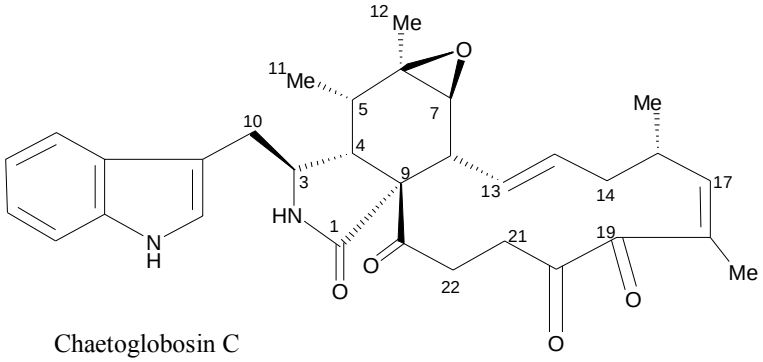


Figure 5.62: AntiMarin search profile for F9245-2



Chaetoglobosin A



Chaetoglobosin C

Figure 5.63: Chaetoglobosin A and chaetoglobosin C

Chaetoglobosin A and chaetoglobosin C have the same mass and resemble each other except in the region between C19 and C22 where the olefinic protons at position H-21 and H-22 in chaetoglobosin A are replaced by methylene protons in chaetoglobosin C and a hydroxyl moiety at position C-19 in chaetoglobosin A is now a carbonyl group in chaetoglobosin C.

To resolve the structure of F9245-2 the COSY NMR spectrum of F9245-2 was recorded (**Figure 5.64**). This showed that the olefinic proton at δ_H 5.01 was coupled to another olefinic proton at δ_H 6.02 (**Figure 5.65a**) and that two other olefinic protons at δ_H 6.10 and 7.23 were also coupled (**Figure 5.65b**)

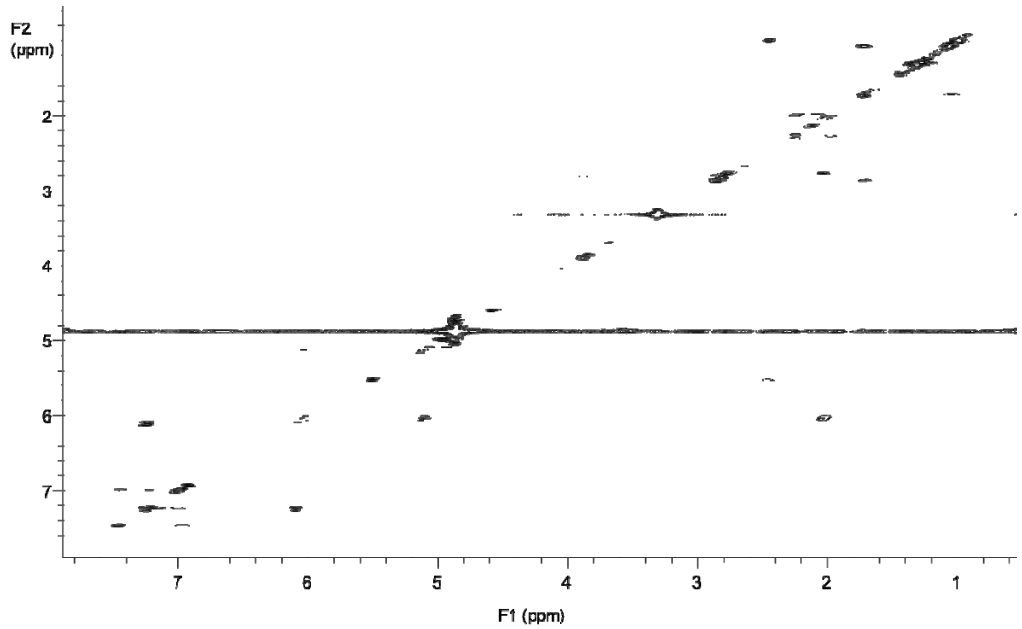


Figure 5.64: COSY NMR spectrum of F9245-2 in CD_3OD.

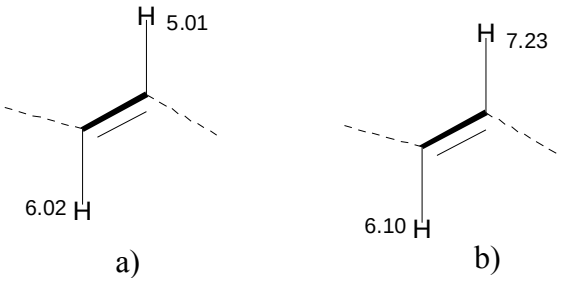


Figure 5.65: Spin systems of F9245-2 obtained by COSY (solid line).

The presence of these four olefinic protons was consistent with the Δ^{13} and Δ^{21} alkene in chaetoglobosin A. A comparison of proton chemical shifts of F9245-2 with the reported data for chaetoglobosin A (Sekita *et al*. 1973) and chaetoglobosin C (Sekita *et al*. 1976) confirmed the structure assignment of F9242-2 as being chaetoglobosin A.

Part C: *Xylaria* sp.

5.10 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 770_3@30.8 originating from a soil sample obtained from inside a cotton tree stump, Eves bush, Nelson and isolated from a 3% phenol plate was identified as belonging to the family Xylariaceae. The colony reached 40 mm diameter after 10 days incubation at 20 °C on MYPA medium. The young colony was initially white with a regular margin (**Figure 5.66a**), slowly turning dark green to black around at the centre after 20 days incubation (**Figure 5.66b**). Stromata initiated in 30 day old culture (**Figure 5.66c**), clavate to cylindrical, mostly unbranched, reaching height of 2 cm or more (**Figure 5.66d**), grayish black with white apices, bearing conidiophores and conidia except at apices. Conidiophores arising as short branch, swollen 5-6 x 3 µm, conidia ellipsoidal 5-6 x 2-3 µm (**Figures 5.66e** and **5.66f**).

As these features correlated very closely with the description of *Xylaria* sp., as provided by Chacko and Rogers (1981), Roger (1986), Callan and Rogers (1993) and Liu *et. al* (2008), fungal strain 770_3@30.8 was identified as *Xylaria* sp.

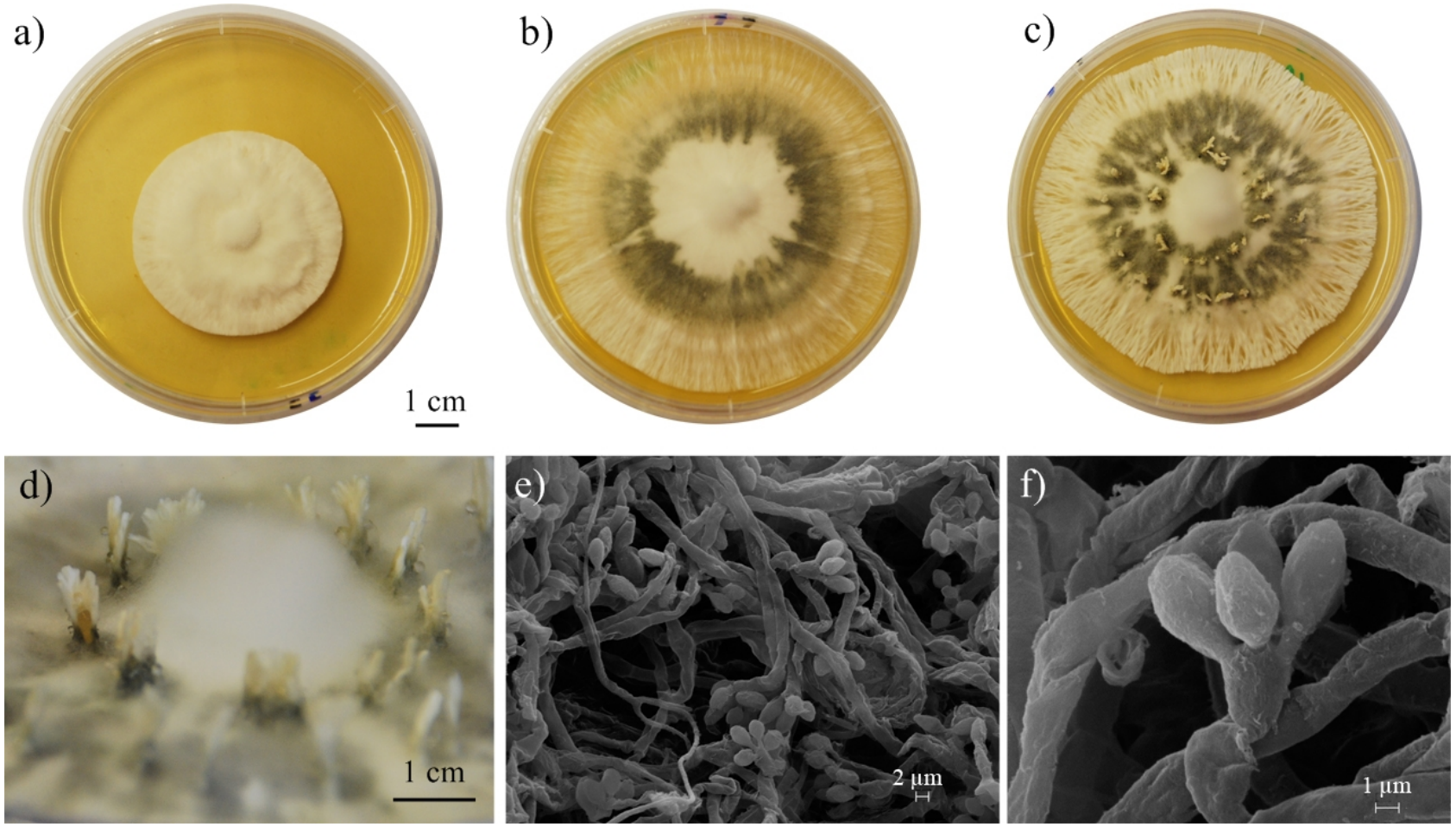


Figure 5.66): *Xylaria sp*. at 20 °C on MYPA plate **a)** 10 days culture; **b)** 20 days culture, center area turning dark green; **c)** 30 days culture with stromata visible on plate; **d)** enlargement of stromata with clear exudates observed; **e-f)** mycelia and conidiophores.

5.11 EFFECT OF ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.11.1 Effect of elicitors on the growth of *Xylaria* sp.

Xylaria sp. was grown on MYPA and SDA at 20 °C for 20 days. The colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of the mycelial growth in the presence of elicitors in MYPA and SDA media is shown in **Figures 5.67** and **5.68** respectively. Stromata development was observed and is presented in **Table 5.14**

Table 5.14: Observation of ascomata production at 20 days incubation.

Elicitors	Development of stromata	
	MYPA	SDA
Control	++	++
TTC	++	+++
MPA	++	+++
TCZ	+	++
NYS 10 U	+	+
NYS 1 U	++	++
CLY 10 µM	N/O	N/O
CLY 5 µM	+	+
CLY 2.5 µM	+	+
PHA 0.1U	++	++
LB 0.5 µg	+++	+++
LB 0.25 µg	+++	+++
JAS 0.05 µM	++	+
JAS 0.01 µM	++	+
HDACI IV 1 µM	+	+
HDACI IV 0.5 µM	N/O	N/O
HDACI IV 0.05 µM	N/O	N/O
HDACI II 0.8 µM	N/O	N/O
HDACI II 0.4 µM	N/O	N/O
HDACI II 0.2 µM	N/O	N/O

* +++ visible stromata observed on plate, ++ entire centre area of colony turned grey indication for developing of ascomata, + centre area of colony partially turned grey, N/O no sign of stromata development.

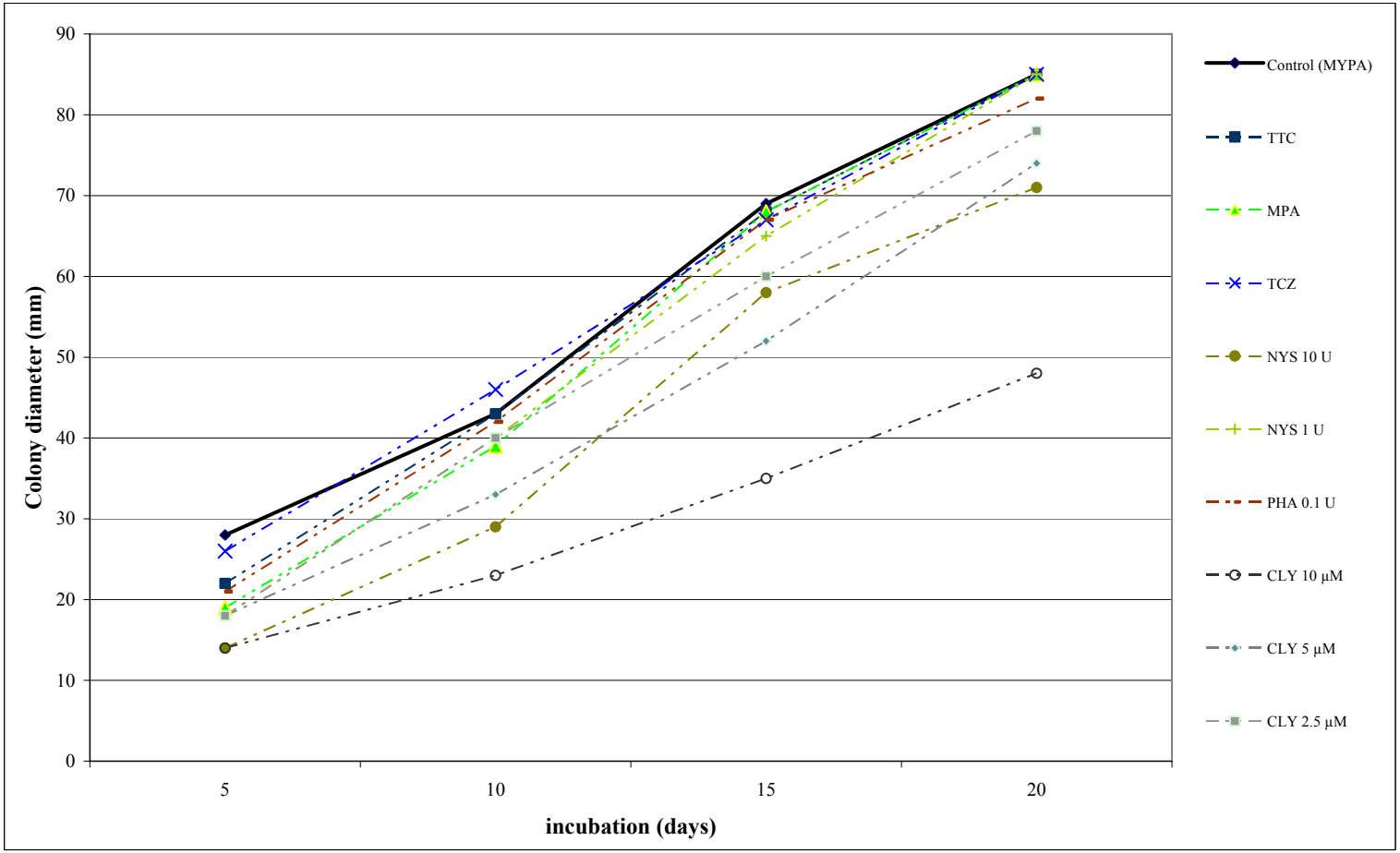


Figure 5.67: Effect of elicitors on growth of *Xylaria* sp. on MYPA at 20 °C

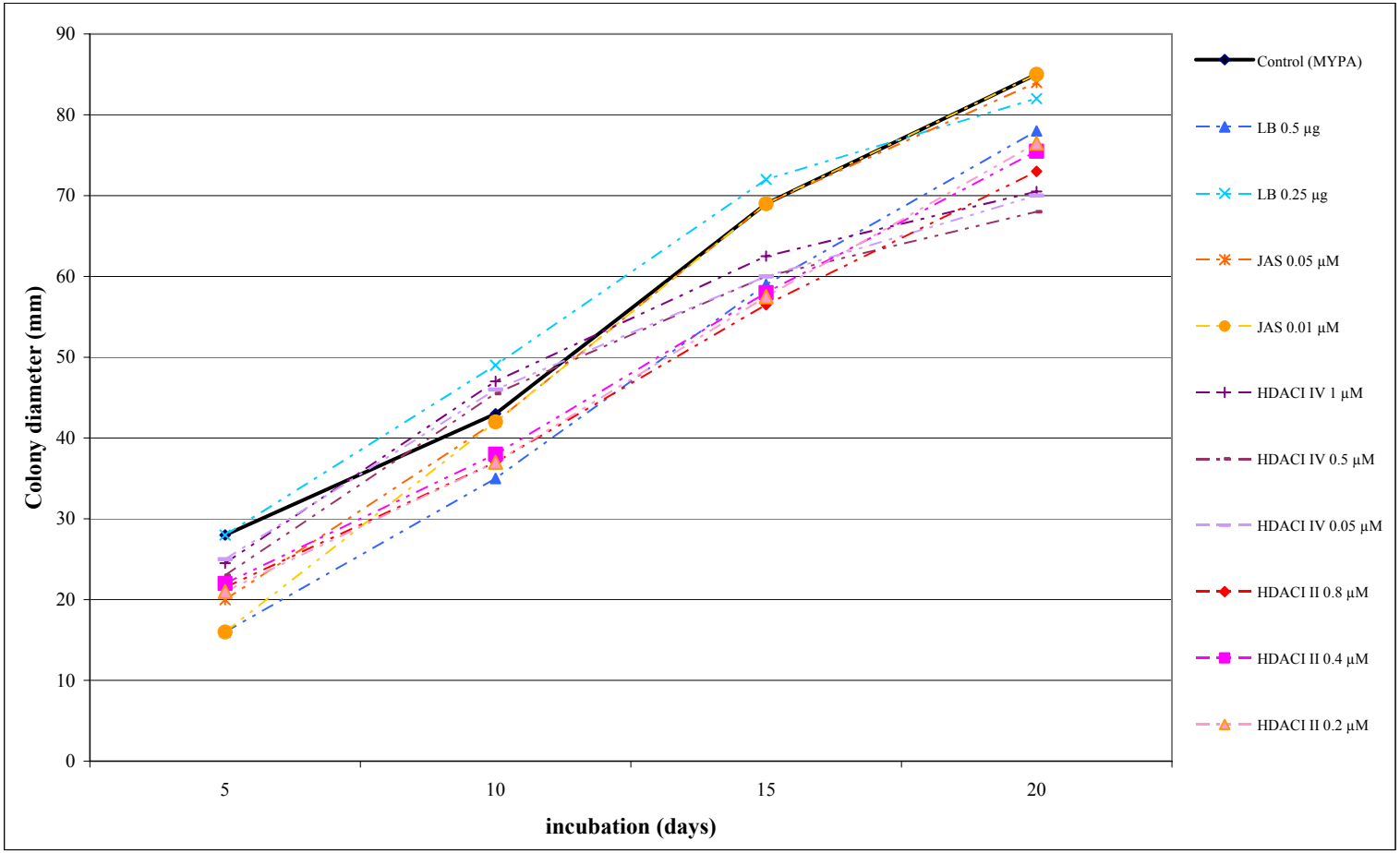


Figure 5.67: (Continued)

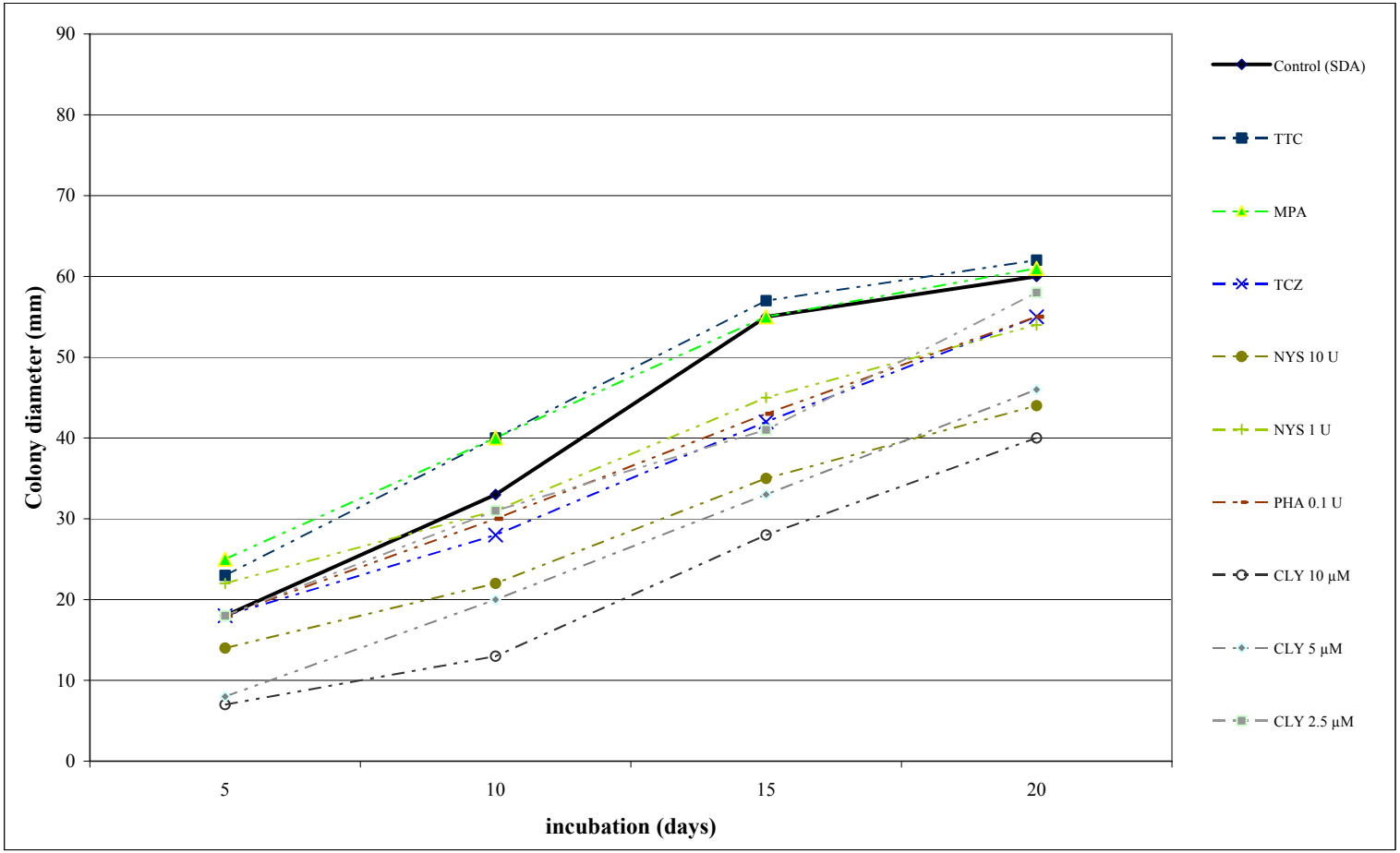


Figure 5.68: Effect of elicitors on growth of *Xylaria* sp. on SDA at 20 °C

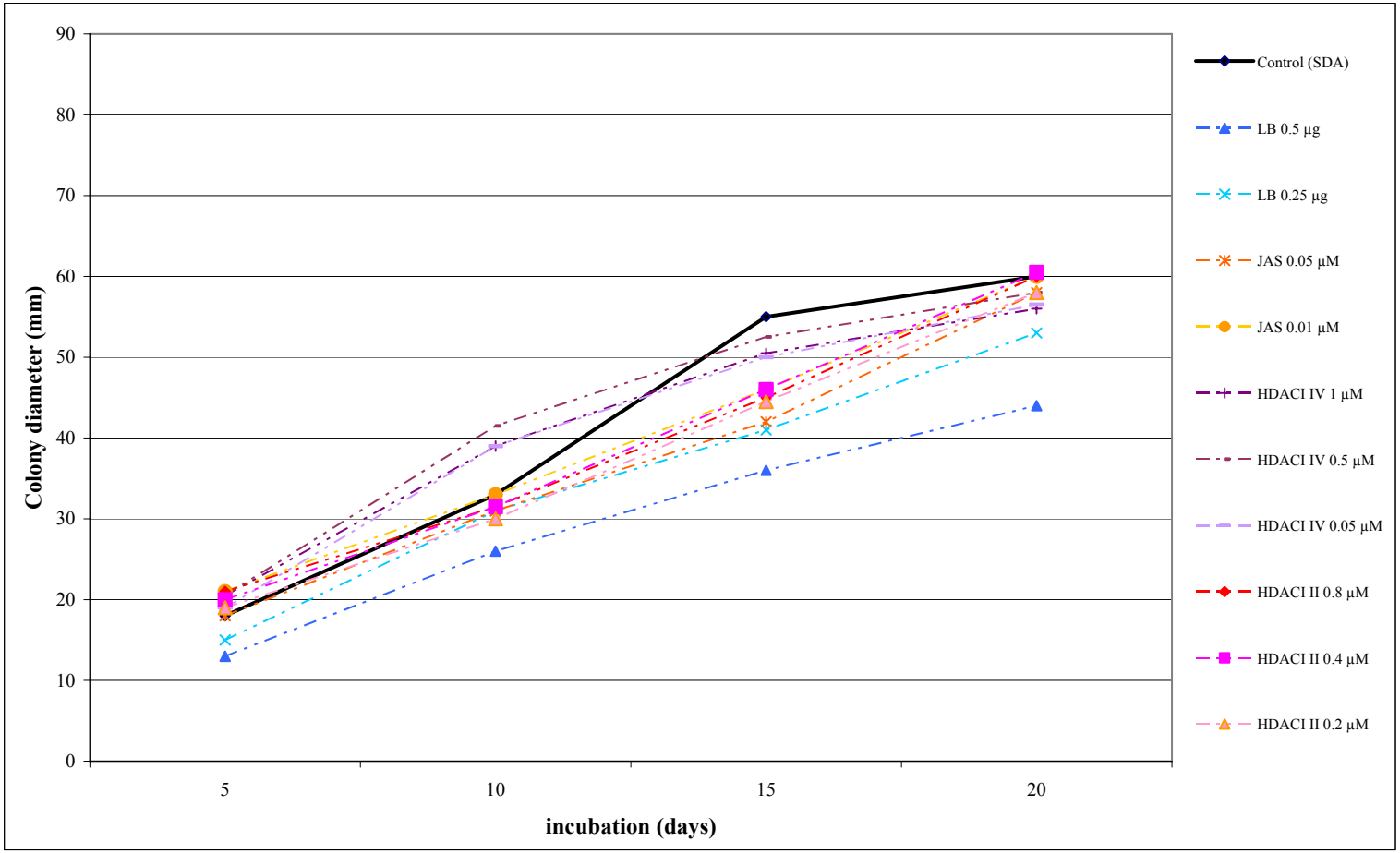


Figure 5.68: (Continued)

It was noted that no growth was observed in the presence of nystatin (NYS) (100 U) in either medium. Without elicitors, growth of the fungus was greater on MYPA than SDA. Due to a slow development of stromata, which appeared on the plate in 30-40 day old cultures, most of the observation of ascomata production (**Table 5.14)** was based on the dark green area at the centre of colony. This is an indication of an initiation of stromata. Nevertheless, stromata were visible on plates in the presence of latrunculin B (LB) at both of the concentrations used. Growth of the fungus was not significantly affected by elicitors except in the presence of cycloheximide (CLY) (10 μM) where the growth was suppressed. Stromata development of this fungus was suppressed in the presence of CLY (10 μM), HADCI IV (0.5 and 0.05 μM), and for all concentrations of HADCI II used in the media.

5.11.2 Effect of the elicitors on metabolite production

5.11.2.1 Effect of the elicitors on the cytotoxicity of extracts

Greater cytotoxicity was shown from extracts obtained from the SDA culture than those obtained on MYPA. Details of the IC_{50} results are shown in **Table 5.15**.

Table 5.15: Effect of elicitors on the cytotoxicity of the extracts from *Xylaria* sp. on MYPA and SDA (20 °C for 20 days).

Elicitors	Cytotoxicity on both media			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9300	10,713	F9315	5,384
TTC	F9301	>12,500	F9316	1,500
MPA	F9302	>12,500	F9317	1,035
TCZ	F9303	>12,500	F9318	11,376
NYS 10 U	F9305	>12,500	F9320	8,431
NYS 1 U	F9306	>12,500	F9321	9,224
CLY 10 µM	F9307	<975	F9322	<975
CLY 5 µM	F9308	>12,500	F9323	<975
CLY 2.5 µM	F9309	>12,500	F9324	<975
PHA 0.1 U	F9310	>12,500	F9325	<975
LB 0.5 µg	F9311	>12,500	F9326	<975
LB 0.25 µg	F9312	>12,500	F9327	4,916
JAS 0.05 µM	F9313	>12,500	F9328	4,172
JAS 0.01 µM	F9314	>12,500	F9329	5,220
HDACI IV 1 µM	F9463	>12,500	F9470	8,834
HDACI IV 0.5 µM	F9465	9,054	F9472	>12,500
HDACI IV 0.05 µM	F9467	4,916	F9474	9,054
HDACI II 0.8 µM	F9554	>12,500	F9560	>12,500
HDACI II 0.4 µM	F9556	>12,500	F9562	>12,500
HDACI II 0.2 µM	F9558	>12,500	F9564	>12,500

5.11.2.2 HPLC screening of extracts

A comparison of ELSD traces (**Figures 5.69** and **5.70)** showed that *Xylaria* sp. produced more metabolites when grown on SDA than on MYPA medium. Compound F9307-1 was induced in the presence of CLY (10 µM), HDACI IV (0.5 µM) and HDACI IV (0.05 µM) in MYPA.

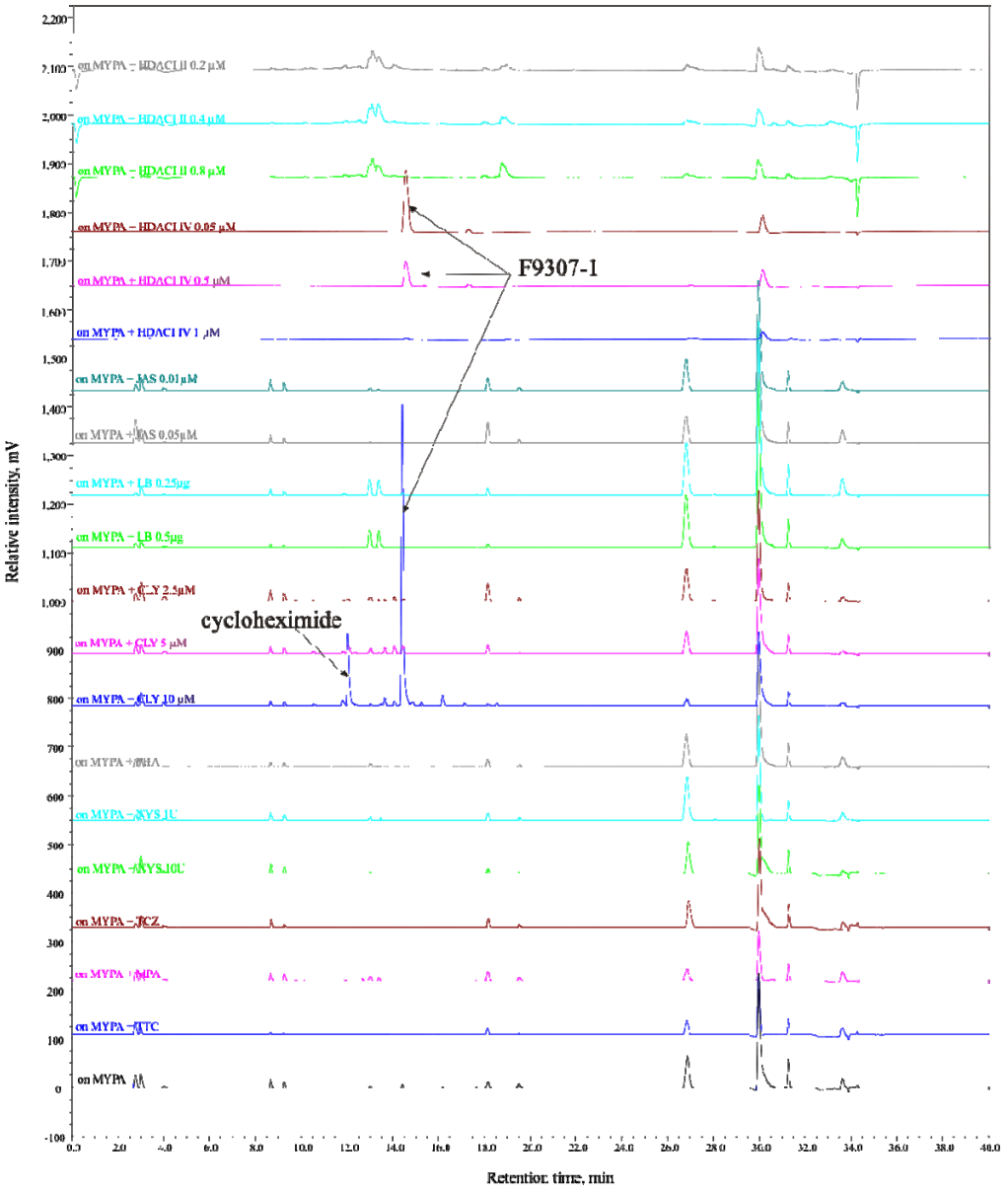


Figure 5.69: HPLC traces of extracts from *Xylaria* sp. on MYPA (20°C for 20 days) with various elicitors.

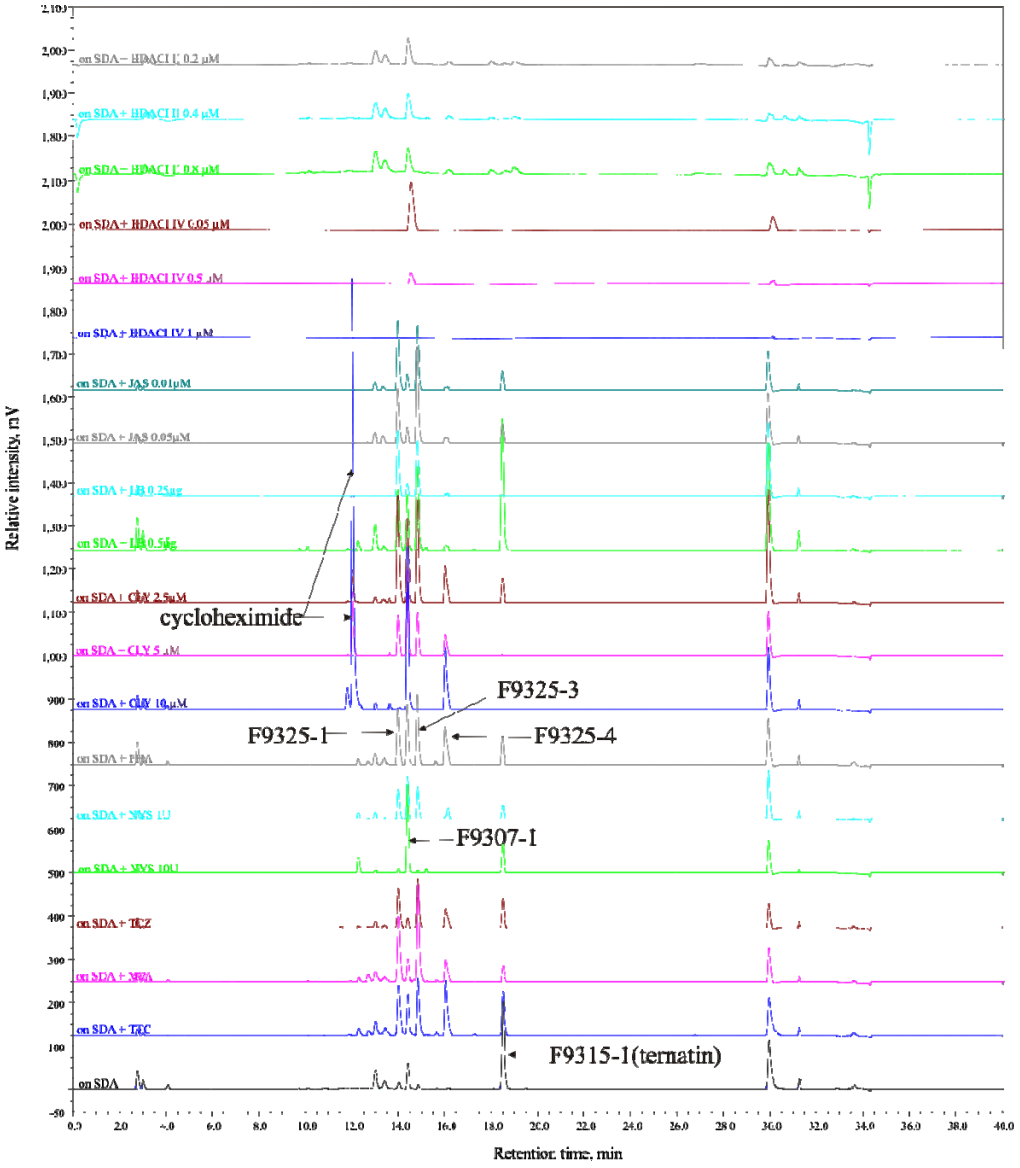


Figure 5.70: HPLC traces of extracts from *Xylaria* sp. on SDA (20°C for 20 days) with various elicitors.

Compound F9315-1 was the main compound produced when the fungus was grown on SDA medium. The production of F9315-1 was suppressed by all elicitors except LB (0.5 µg) (see **Figure 5.70**).

Four metabolite peaks in the region 13.0-17.0 min were enhanced when certain elicitors were present in the medium as summarized in **Table 5.16**.

Metabolite production was totally suppressed in the presence of HDACI IV (1µM). The main metabolites are shown in **Table 5.16** and the structural elucidation is discussed in **Section 5.12**.

Table 5.16: Metabolites produced in presence of elicitors.

Summary of metabolite peaks					
Metabolites	**Peak name**	**Elution (min)**	**Media**	**Elicitors**	**Note**
Ternatin	F9315-1	16.34	SDA	none	Original peak
				LB 0.5 µg	Enhanced peak
2-hexylidene-3-methlysuccinic acid	F9307-1	14.37	MYPA	CLY 10 µM HDACI IV 0.5 µM HDACI IV 0.05 µM	Induced peak
			SDA	TTC NYS 10, 1U CLY 10, 5, 2.5 µM PHA 0.1 U LB 0.5 µg HDACI IV 0.05 µM	Enhanced peak
Dechlorogriseofulvin	F9325-1	13.99	SDA	TTC MPA TCZ NYS 1U CLY 5, 2.5 µM PHA 0.1 U LB 0.5, 0.25 µg JAS 0.05, 0.01 µM	Enhanced peak
Griseofulvin	F9325-3	14.83	SDA	TTC MPA TCZ NYS 1U CLY 10, 5, 2.5 µM PHA 0.1 U LB 0.5, 0.25 µg JAS 0.05, 0.01 µM	Enhanced peak
Chaetoglobosin E	F9325-4	16.02	SDA	TTC MPA TCZ CLY 10, 5, 2.5 µM PHA 0.1 U	Induced peak

5.12 STRUCTURAL ELUCIDATION OF COMPOUNDS PRODUCED BY *Xylaria* sp.

5.12.1 Peak F9315-1

The crude extract of F9315 was screened by HPLC microtitre plate collection and the ELSD HPLC chromatogram showed the presence of only one main peak which eluted at 16.3 min, F9315-1 (**Figure 5.71**).

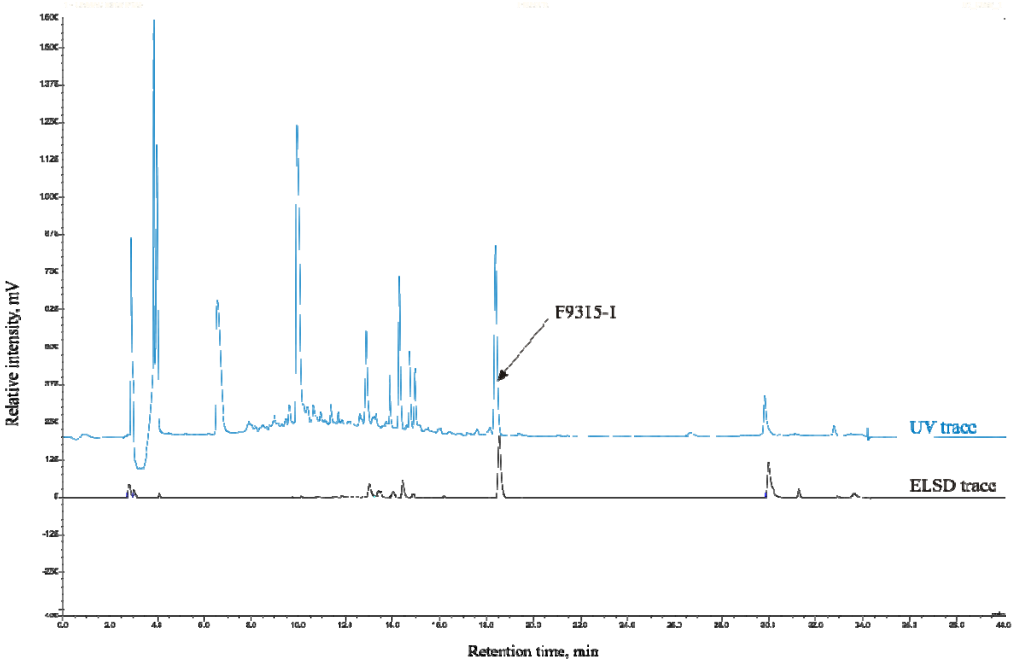


Figure 5.71: HPLC chromatogram of crude extract F9315. UV detection (blue) with ELSD (black) comparison.

A search of the HPLC-UV/R_t library database for the UV chromorphores and R_t of this peak showed matches with ternatin (**Figure 5.72**).

	Name	Match	Library	Ret.Time[mi	ID	#Extrema
1	ternatin	999.05	Known active	17.885	A-117	0
2	T987B	997.51	Known active	17.875	A-33	1
3	T987A	993.47	Known active	18.297	A-120	0
4	M-6124	991.12	Known active	17.530	A-34	1
5	Cytochalasin Q	990.15	Known active	15.941	A-35	0
6	bassiatin	990.05	Known active	14.541	A-95	0
7	cytochalasin D	989.44	Known active	14.843	A-172	0
8	cyclo-(LPhe-transL4Hyp)	987.75	Known active	9.108	A-53	0
9	cytochalasin J	986.48	Known active	12.837	A-411	0
10	Deacetylzygosporin G	985.25	Known active	16.248	A-47	0
11	Hirsutide	982.97	Known active	17.830	A-48	0
12	cyclo(L pro L val)	980.48	Known active	8.113	A-111	0
13	Beauvericin A	976.94	Known active	26.047	A-14	1
14	Beauveriolide (AM 25681)	976.83	Known active	17.000	A-7	0

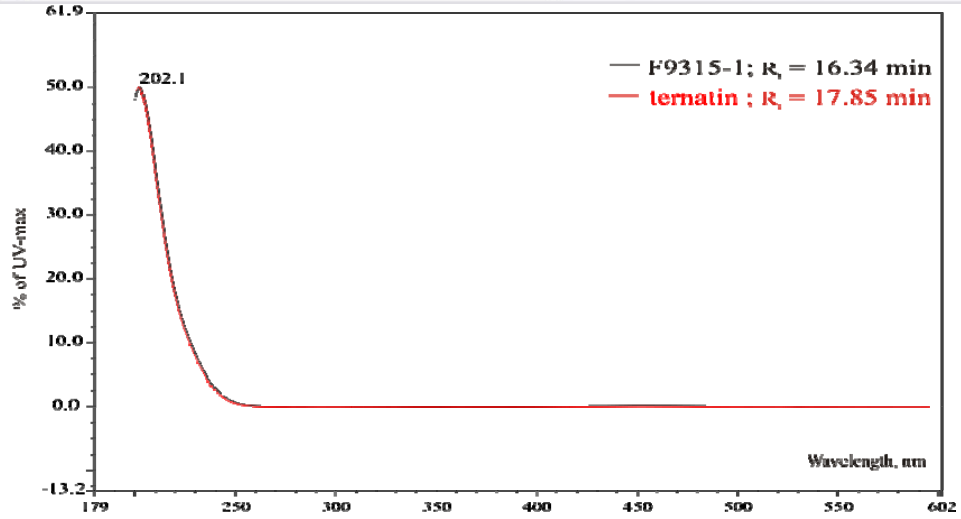


Figure 5.72: Extracted UV spectra of peak F9315-1 (black) and ternatin (red) from search in the HPLC-UV/R_t library database.

ESIMS analysis showed that compound F9315-1 had a molecular mass ([M+H^+]) of 738.9 (**Figure 5.73**) which matched that of ternatin (Shimokawa *et al*. 2006). Ternatin was reported to exhibit an antiviral activity as well as a moderate cytotoxicity (Simões *et al*. 1990).

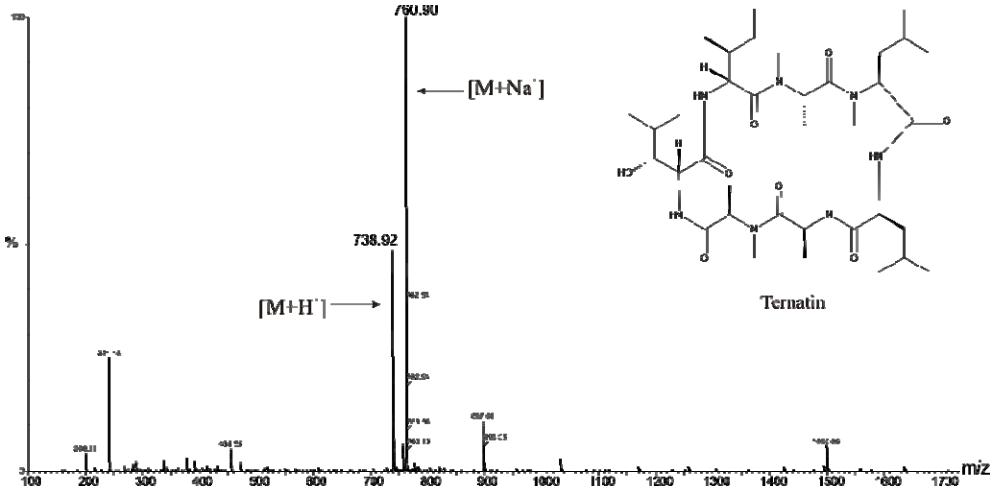


Figure 5.73: ESIMS spectrum of compound F9315-1 assigned as ternatin.

5.12.2 Peak F9307-1

The HPLC profile of the extract F9307 obtained from MYPA containing cycloheximide showed the presence of cycloheximide in the extract (R_t 12.0 min) and one other metabolite peak on ELSD trace, F9307-1 (**Figure 5.74**).

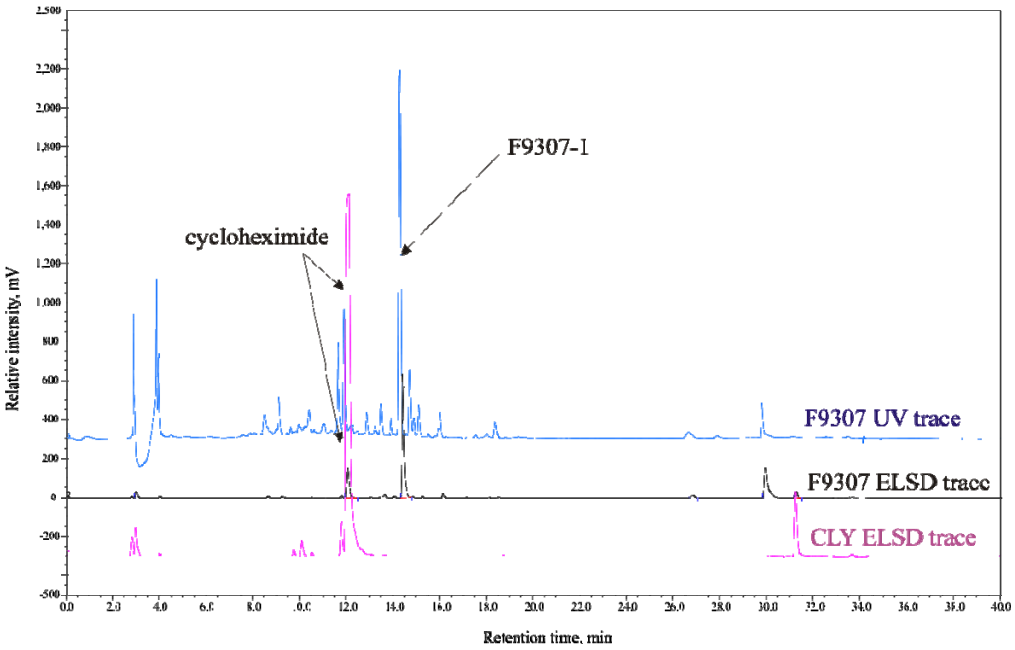


Figure 5.74: HPLC chromatograms of cycloheximide (pink) and crude extract F9307 UV detection (blue) with ELSD (black) comparison.

The UV chromophore of the peak that eluted at 12.0 min from F9307 was synchronized with the UV chromophore of cycloheximide (**Figure 5.75**), and thereby confirmed the identity of the peak eluting at 12.0 min as being cycloheximide.

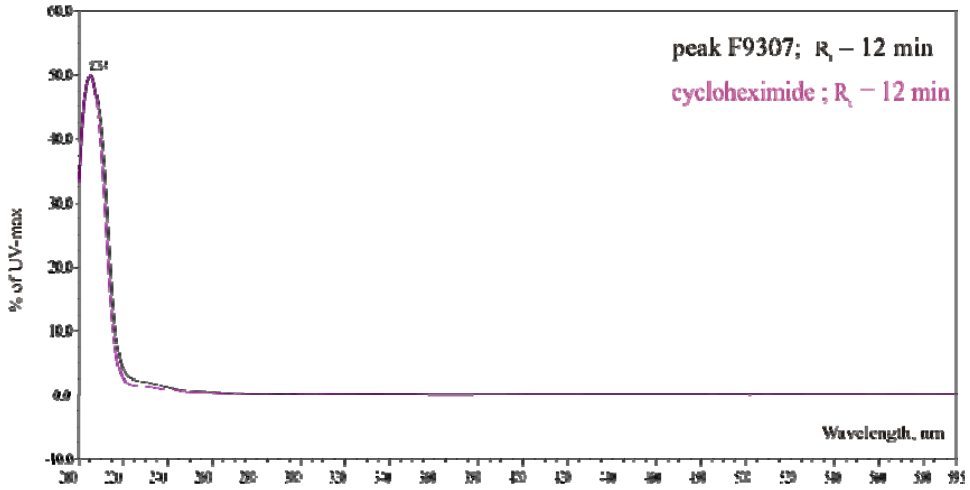


Figure 5.75: Extracted UV spectra of peak at 12.0 min from F9307 (black) cycloheximide (pink).

A search of the HPLC-UV/R_t library database and the UV profile of the peak at R_t = 14.37 min showed a match with 2-hexylidene-3-methylsuccinic acid (**Figure 5.76**).

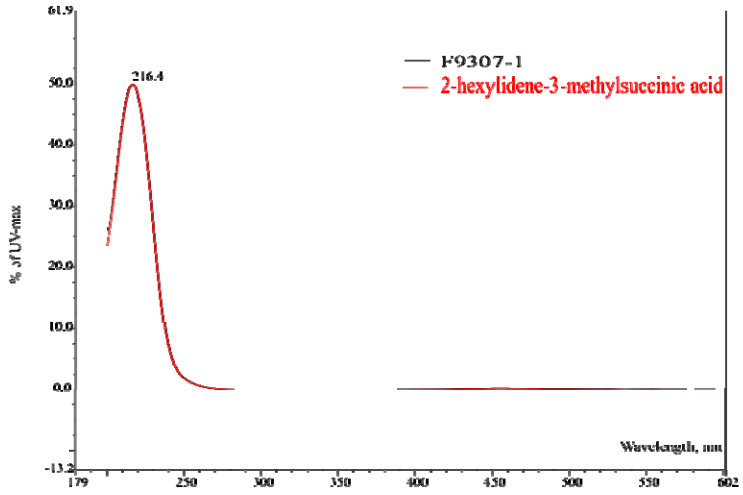


Figure 5.76: Extracted UV spectra of peak F9307-1 (black) and 2-hexylidene-3-methylsuccinic acid (red).

ESIMS showed that this peak has a molecular mass of 215 amu ([M+H$^+$]) (**Figure 5.77**) matching that of 2-hexylidene-3-methylsuccinic acid (Anderson *et al*. 1985; Liu *et al.* 2006).

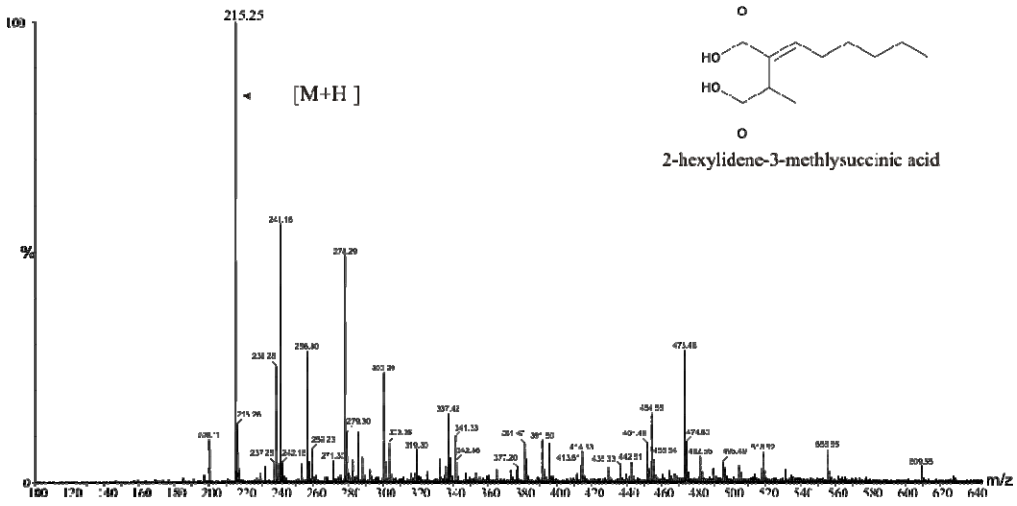


Figure 5.77: ESIMS spectrum of F9307-1.

5.12.3 Extract F9325

HPLC analysis of the F9325 extract revealed the presence of five main compounds in the ELSD trace; F9325-1, F9325-2, F9325-3, F9325-4 and F9325-5 appearing at R_t 13.0-16.5 min (**Figure 5.78**). Peaks F9325-2 and F9325-5 were identified as 2-hexylidene-3-methylsuccinic acid and ternatin respectively (see **Sections 5.12.1** and **5.12.2** for details).

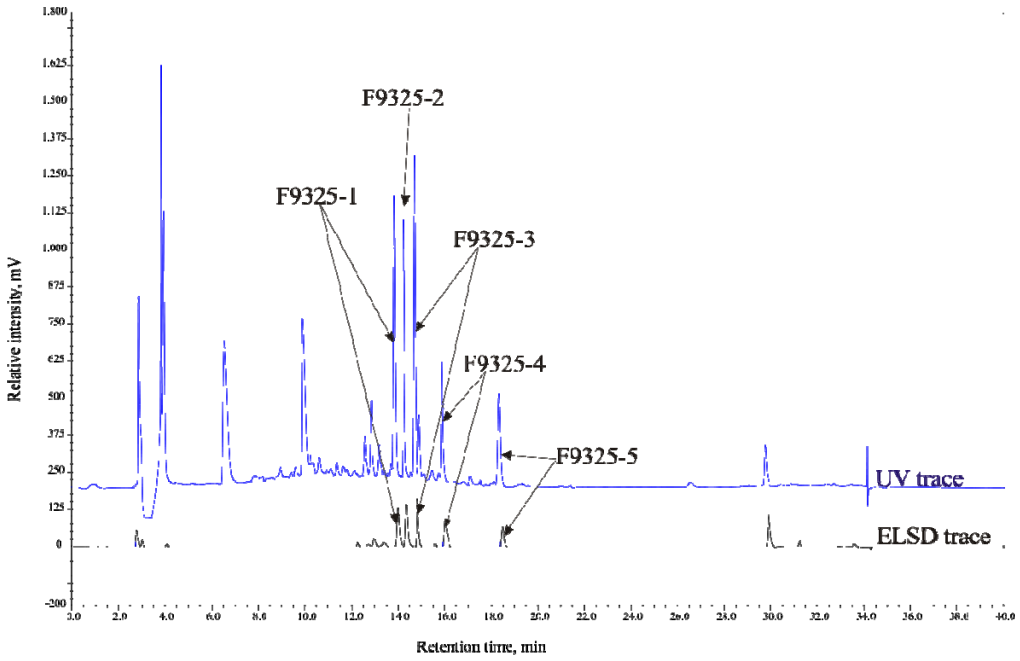


Figure 5.78: HPLC chromatogram of crude extract F9325. UV detection (blue) with ELSD (black) comparison.

5.12.3.1 *Peak F9325-1*

The UV profile of peak F9325-1 (**Figure 5.79**) showed an exact match with dechlorogriseofulvin in the HPLC-UV/R_t library database.

	Name	Match	Library	Ret.Time[mi	ID	#Extrema
1	dechlorogriseofulvin	998.60	Known active	13.707	A-80	3
2	Griseofulvin	956.17	Known active	14.523	A-1	4
3	Paecilosetin	897.92	Known active	20.740	A-15	4
4	Epicoccamide	852.35	Known active	19.017	A-227	3
5	radicicol	845.77	Known active	13.345	A-122	3
6	WF-3681	834.23	Known active	9.910	A-148	3
7	Chloramphenicol	823.23	Known active	11.742	A-59	2
8	Curvulinic acid	819.51	Known active	8.261	A-415	3
9	Mono-o-methylcurvulinic acid	816.17	Known active	10.236	A-416	4
10	Oosporein	813.07	Known active	10.121	A-49	3
11	2,4diOH-3Me-6-(2-oxo-propyl)benzaldeh	809.77	Known active	12.231	A-257	4
12	bikaverin	805.85	Known active	16.723	A-96	4
13	A Bisnaphthopyrone (F3225)	799.62	Known active	20.412	A-31-1	5
14	equisetin	799.24	Known active	24.441	A-159	3

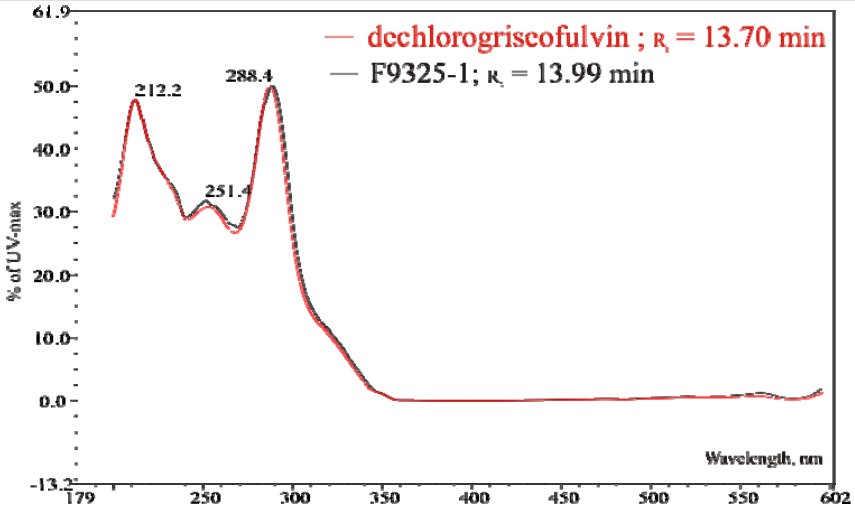


Figure 5.79: Extracted UV spectra of peak F9325-1 (black) and ternatin (red) with a search in the HPLC-UV/R_t library database.

ESIMS data for this compound (**Figure 5.80**) indicated that the mass was 319 amu ([M+H^+]), identical to that reported mass for dechlorogriseofulvin isolated from *Penicillium urticae* (Cole *et. al*, 1970).

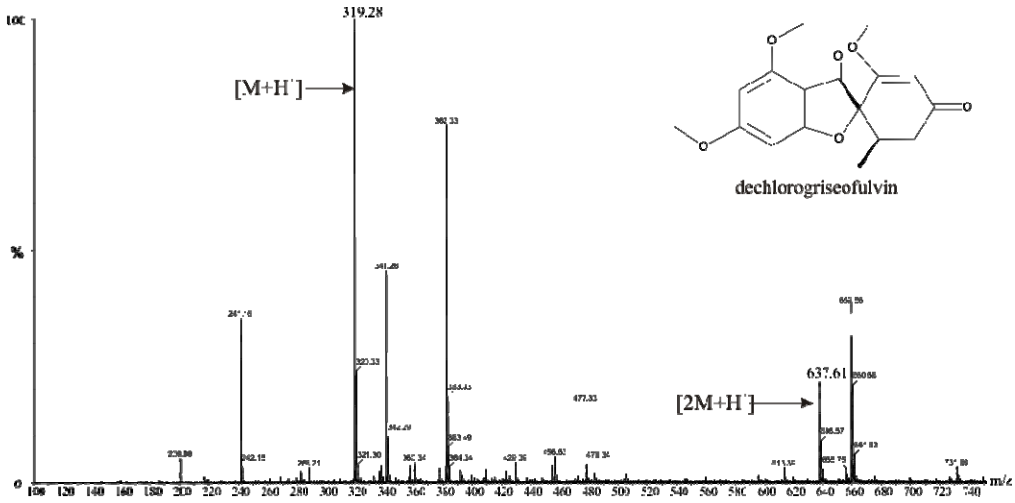


Figure 5.80: ESIMS spectrum of compound F9325-1 determined as dechlorogriseofulvin.

5.12.3.2 *Peak F9325-3*

The extracted UV spectrum of F9325-3 is shown in **Figure 5.81**. A search in the in-house UV database showed that it was likely to be griseofulvin.

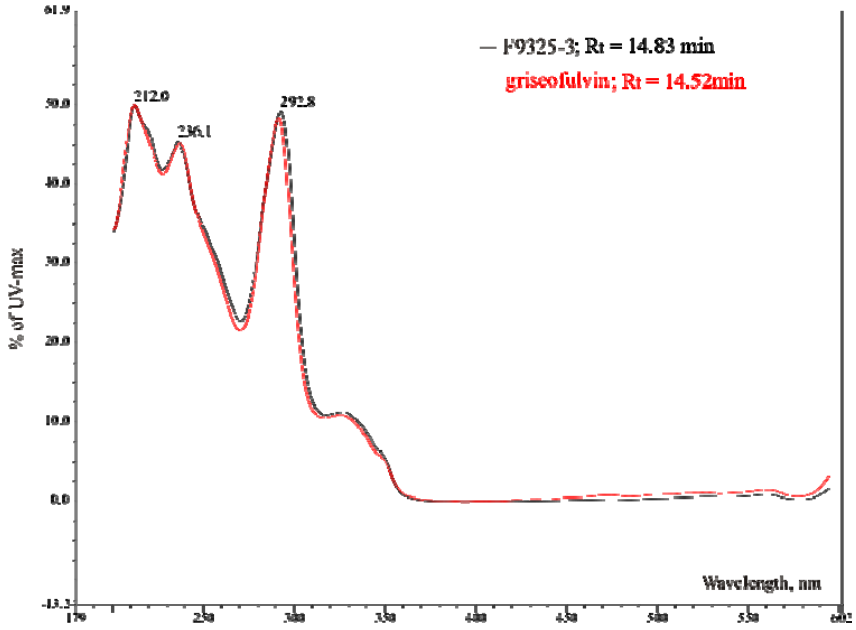


Figure 5.81: Extracted UV spectra of peak F9325-3 (black) and griseofulvin (red) with a search in the HPLC-UV/R_t library database.

ESIMS data for this compound (**Figure 5.82**) indicated the mass to be 353/355 amu (3:1) ([M+H$^+$]). Consistent with griseofulvin (Oxford *et al.*, 1939).

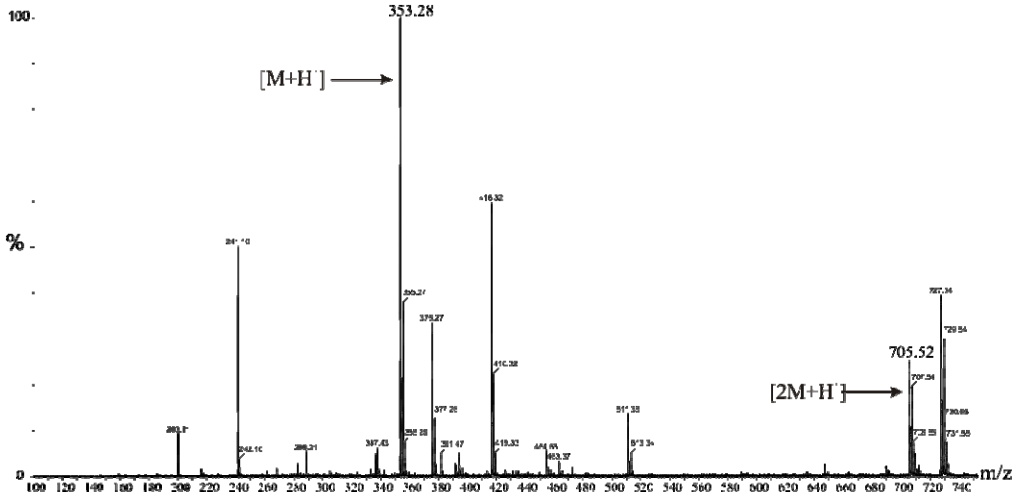


Figure 5.82: ESIMS spectrum of compound F9325-3

The ^{1}H NMR spectrum of F9325-3 was also obtained and it revealed the presence of one doublet methyl, three methoxy protons, a singlet aromatic proton (δ_H 6.4) and one olefinic proton (δ_H 5.6) (**Figure 5.83**).

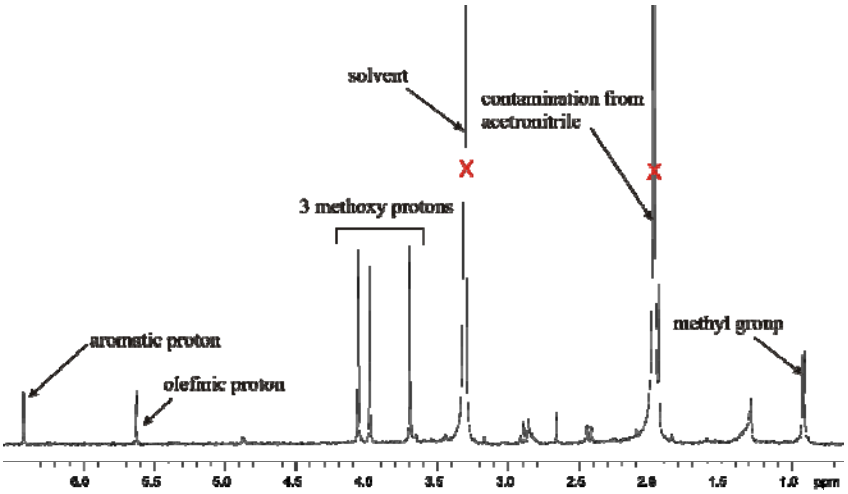


Figure 5.83: ^{1}H NMR spectrum of F7325-3; 15 µg in 6 µL of CD_3OD, recorded at 500 MHz, 167 sec.

These features were identical with those from the ^{1}H NMR spectrum of F8767-1 (see **Chapter 4**, **Section 4.3.2)** which was identified as griseofulvin (**Figure 5.84**).

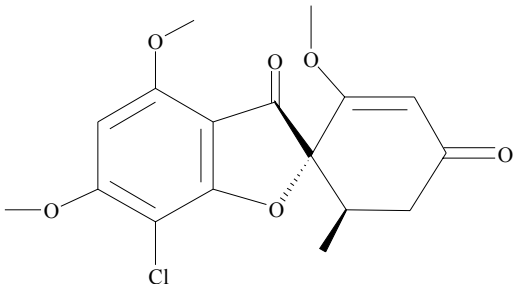


Figure 5.84: Griseofulvin

5.12.3.3 *Peak F9325-4*

The UV profile of compound F9325-4 (**Figure 5.85**) showed no exact match to any compounds in the HLPC-UV/R_t library database and the ESIMS spectrum indicated the molecular mass was 495 amu ([M+H^+]) (**Figure 5.86**).

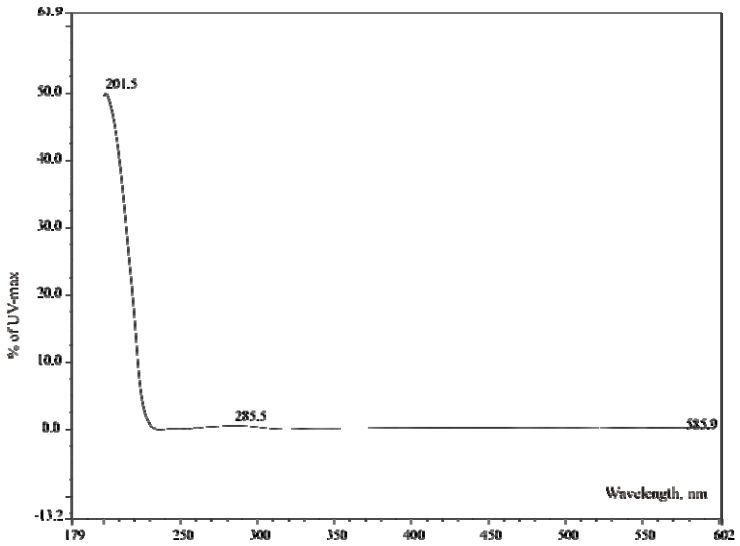


Figure 5.85: Extracted UV spectra of peak F9325-4.

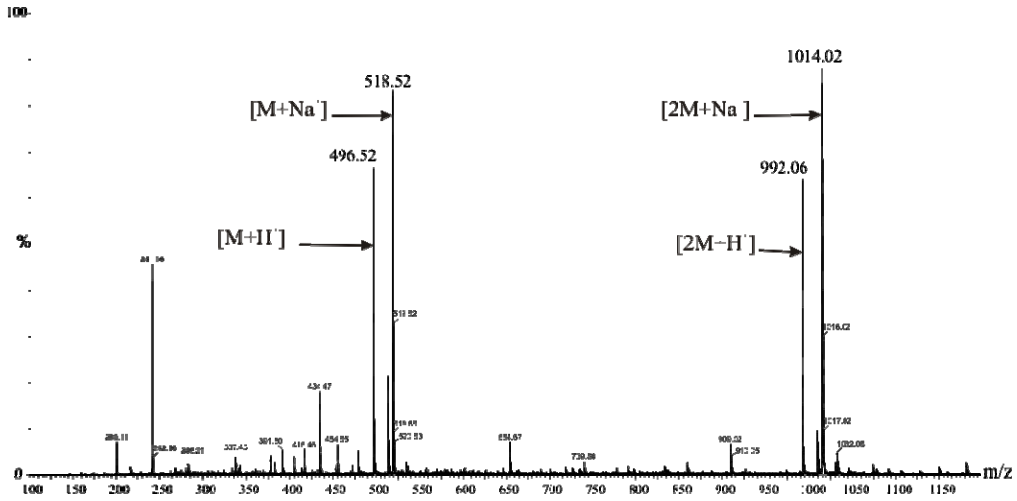


Figure 5.86: ESIMS spectrum of compound F9325-4

The ^{1}H NMR spectrum of F9325-4 revealed the presence of two singlet and two doublet methyls, and one monosubstituted benzene moiety (**Figure 5.87**).

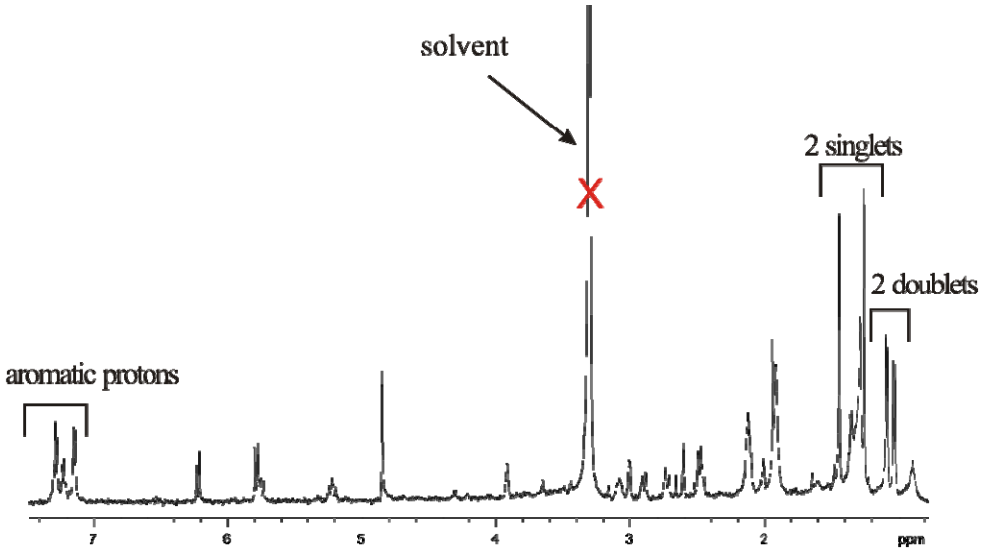


Figure 5.87: ^{1}H NMR spectrum of F7325-3; 15 µg in 6 µL of CD_3OD, recorded at 500 MHz, 191 sec.

A search of the AntiMarin database using a search profile of four methyl groups assigned as two singlets and two doublets, one monosubstituted benzene and a molecular weight of 494-496 amu (**Figure 5.88a**) resulted in a

match with cytochalasin E (**Figure 5.88e**). This cytochalasin had previously been isolated from *Rosellinia necatrix* (Aldridge *et. al*, 1972).

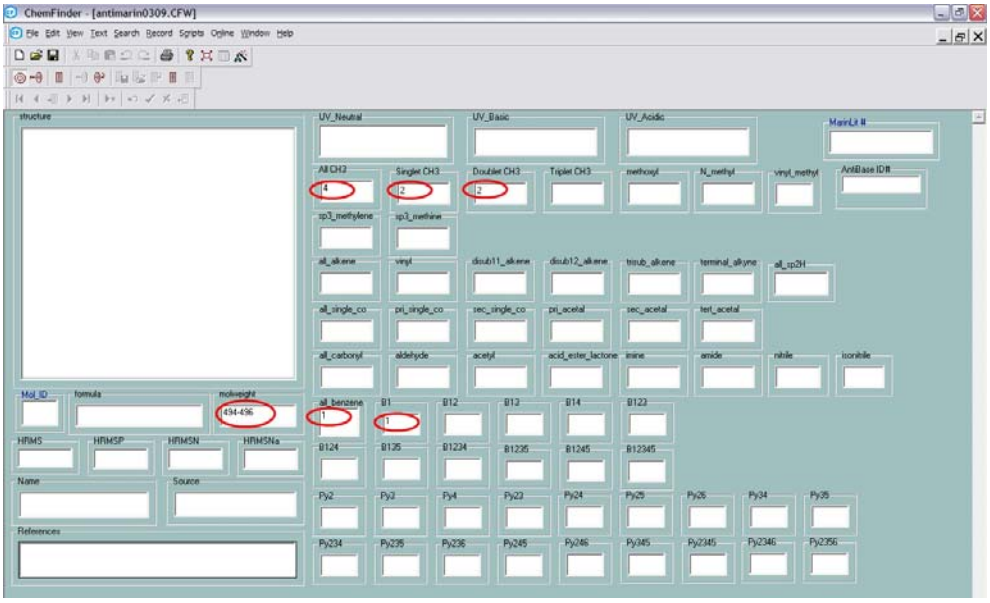


Figure 5.88a: AntiMarin search profile for F9325-4

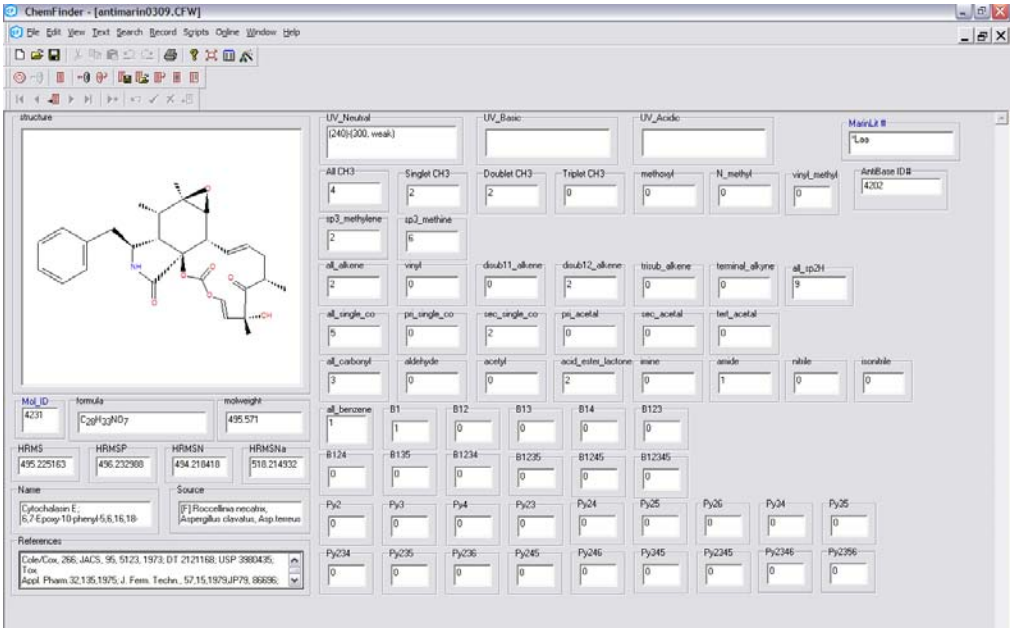


Figure 5.88b: AntiMarin search result for F9325-4

A comparison of the proton chemical shifts of F9325-4 with the reported NMR data of being cytochalasin E (Takamatsu *et. al*, 2002) confirmed F9325-4 as cytochalasin E (**Figure 5.89**).

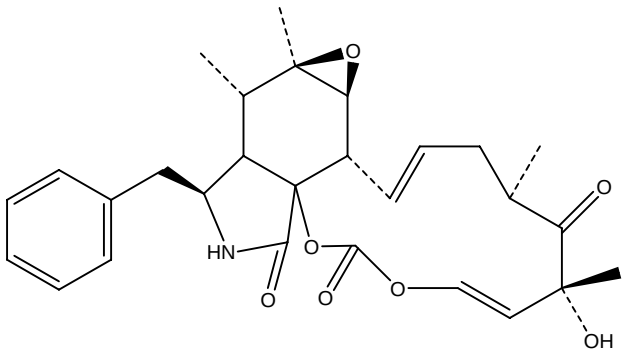


Figure 5.89: Cytochalasin E

Part D: *Bombardia* sp.

5.13 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 902_4@20.5 originated from soil collected from Tiromoana Bush, North Canterbury, and isolated from a 4% phenol plate. This strain was identified as belonging to the family Lasiosphaeriaceae. Colonies on MYPA reached 85 mm in diameter in 10 days at 20 °C. The young colony showed a whitish grey aerial mycelium with light brown gelatinized hyphae adpressed to the agar surface (**Figure 5.90a**). Perithecia are superficial, scattered or aggregated in a loose cluster, dark brown in colour and are produced in abundance on the culture within 15-20 days, (**Figure 5.90b**), spherical or ovate, ostiolate, measuring 450-500 x 250-300 μm (**Figure 5.90c**). Ascospores ovate, 20-35 x 10 μm (**Figure 5.90d**). A hyaline tail of ascospores was observed under a compound microscope, but was evanescent under SEM. A comparison of colony and morphological features with the descriptions provided by Verma (1939), Jensen (1985), Hyde (1993) and Huhndorf *et al.* (2004) enabled strain 902_4@20.5 to be identified as a *Bombardia* sp.

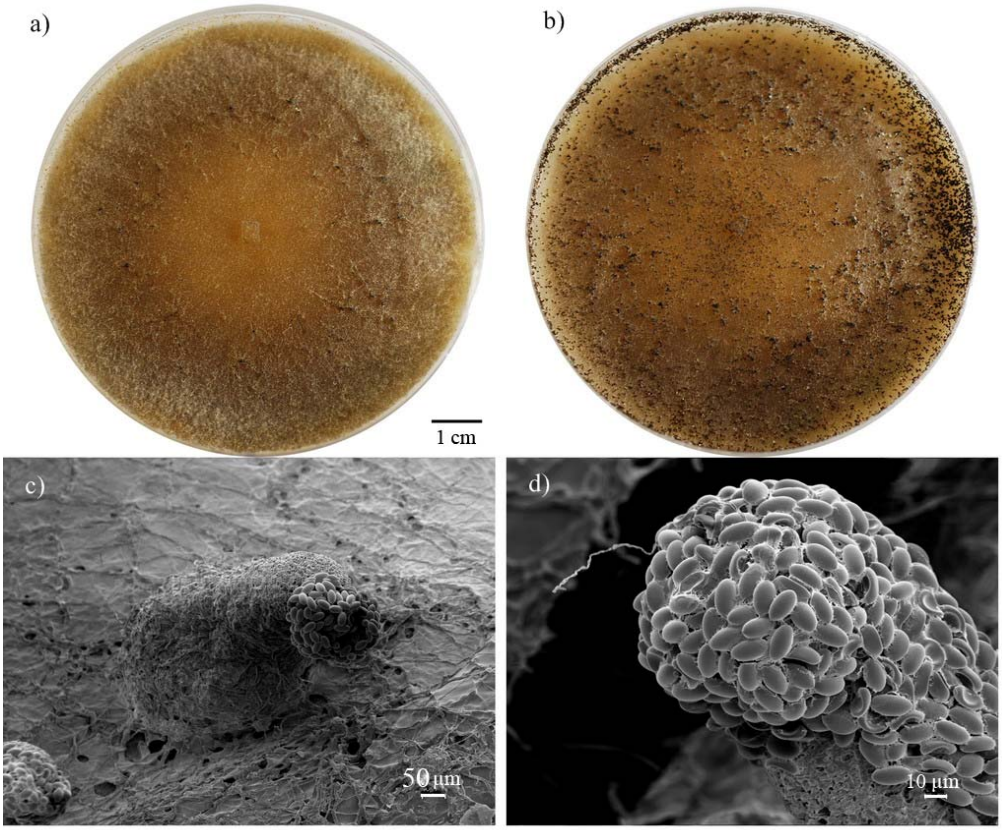


Figure 5.90: *Bombardia* sp. **a)** 10 day colony on MYPA plate; **b)** 20 day colony on MYPA plate with perithecia; **c)** SEM of perithecium **d)** SEM of ascospores.

5.14 EFFECT OF ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.14.1 Effect of elicitors on the growth of *Bombardia* sp.

Bombardia sp. was grown on MYPA and SDA at 20 °C for 20 days. The colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of the mycelial growth in the presence of elicitors in MYPA and SDA is shown in **Figures 5.91** and **Figure 5.92** respectively. Ascocarp production was observed and the data is presented in **Table 5.17**

Table 5.17: Observation of ascomata production at 20 days incubation.

Elicitors	Observation of visible ascocarps	
	MYPA	SDA
Control	++	+++
TTC	+++	++
MPA	+++	++
TCZ	++	++
NYS 1 U	++	+
PHA 0.1U	+++	++
LB 0.5 µg	+	+
LB 0.25 µg	+	+
JAS 0.05 µM	++	++
JAS 0.01 µM	+++	++
HDACI IV 1 µM	+	+
HDACI IV 0.5 µM	++	++
HDACI IV 0.05 µM	++	++
HDACI II 0.8 µM	++	+
HDACI II 0.4 µM	+++	++
HDACI II 0.2 µM	+++	+++

* +++ visible ascomata cover entire colony, ++ visible ascomata abundance at the edge of the colony, + sparse ascomata observed.

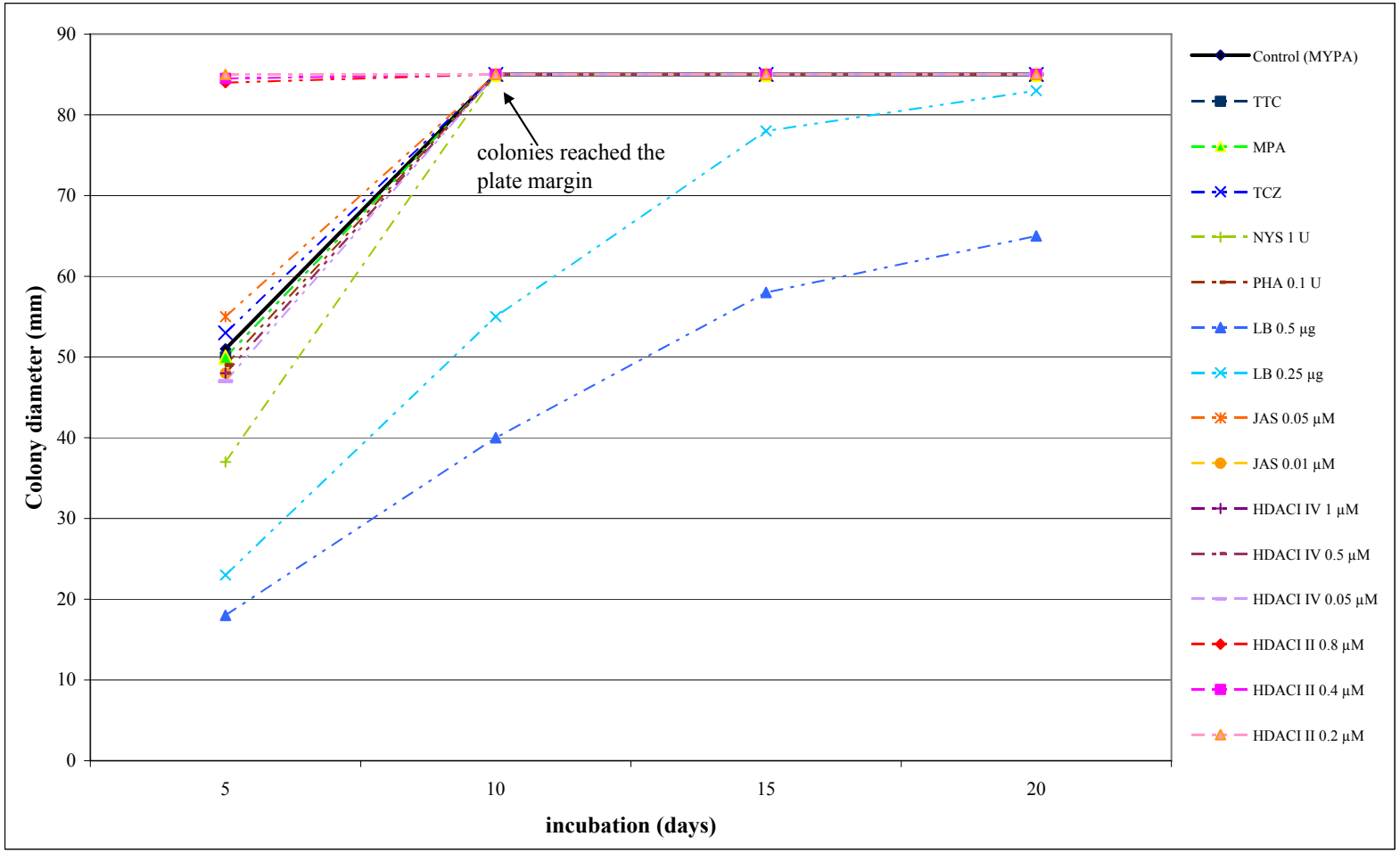


Figure 5.91: Effect of elicitors on the growth of *Bombardia* sp. on MYPA at 20 °C

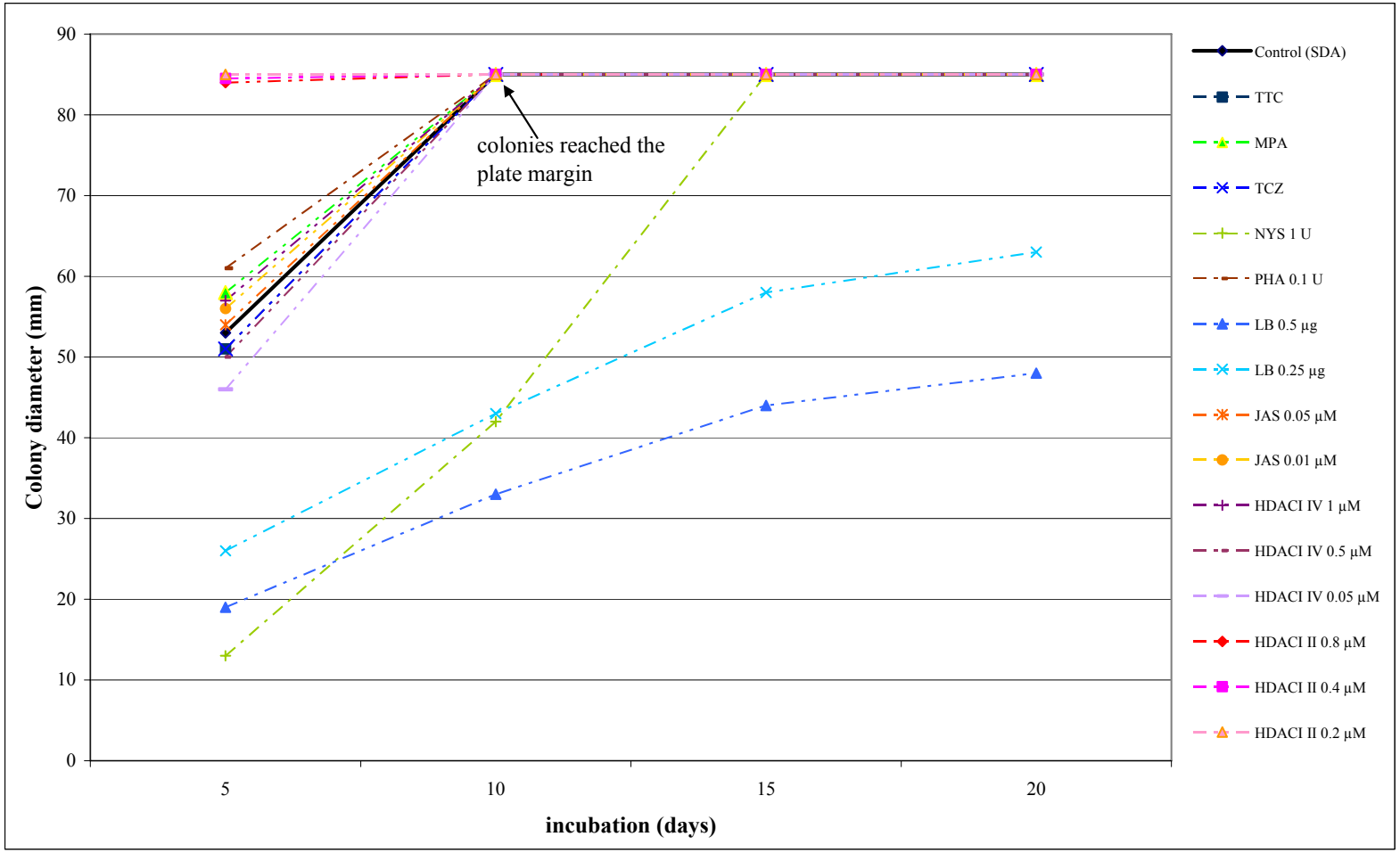


Figure 5.92: Effect of elicitors on the growth of *Bombardia* sp. on SDA at 20 °C

No growth was observed with cycloheximide and nystatin (100 U and 10 U concentrations) in the media. Interestingly, no differences were seen between the two media used. Furthermore, growth on both media was enhanced in the presence of HDACI II (at all concentrations), but was suppressed in the presence of LB at both concentrations (**Figures 5.91** and **5.92**). Ascomata production was not affected by elicitors except in the presence of LB and HDACI IV (1 μM) where perithecial production was reduced.

5.14.2 Effect of the elicitors on cytotoxic and metabolite production

5.14.2.1 Effect of the elicitors on cytotoxicity of extracts

The cytotoxicity of the extracts from each elicitor treatment is shown in **Table 5.18**. Greater cytotoxicity was seen with extracts from MYPA rather than SDA plates and the best activity was seen using MPA as the elicitor.

Table 5.18: Effect of elicitors on the cytotoxicity of extracts from *Bombardia* sp. on MYPA and SDA (20 °C for 20 days).

Elicitors	Cytotoxicity			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9330	11,713	F9345	12,395
TTC	F9331	11,892	F9346	>12,500
MPA	F9332	9,772	F9347	>12,500
TCZ	F9333	9,224	F9348	11,376
NYS 1 U	F9336	11,376	F9351	>12,500
PHA 0.1 U	F9340	9,054	F9355	>12,500
LB 0.5 µg	F9341	11,376	F9356	>12,500
LB 0.25 µg	F9342	11,376	F9357	>12,500
JAS 0.05 µM	F9343	8,639	F9358	>12,500
JAS 0.01 µM	F9344	7,432	F9359	>12,500
HDACI IV 1 µM	F9477	8,431	F9484	>12,500
HDACI IV 0.5 µM	F9479	9,054	F9486	>12,500
HDACI IV 0.05 µM	F9481	8,687	F9488	>12,500
HDACI II 0.8 µM	F9566	9,224	F9572	>12,500
HDACI II 0.4 µM	F9568	8,666	F9574	>12,500
HDACI II 0.2 µM	F9570	7,432	F9576	>12,500

5.14.2.2 *HPLC screening of the extracts*

A comparison of the ELSD traces (**Figures 5.94** and **5.95**) showed that *Bombardia* sp. produced a range of metabolites when grown on MYPA but none with SDA. On MYPA, eight metabolite peaks; F9332-1 to F9332-8, were induced by all elicitors except LB at both concentrations (see **Figure 5.93**). An enhancement of peaks in the region 8.0-9.5 min was observed in the presence of certain elicitors; TTC, MPA, JAS (0.01 µM), HDACI IV (1 and 0.05 µM) and HDACI II (0.4 and 0.2 µM) (**Figure 5.93**).

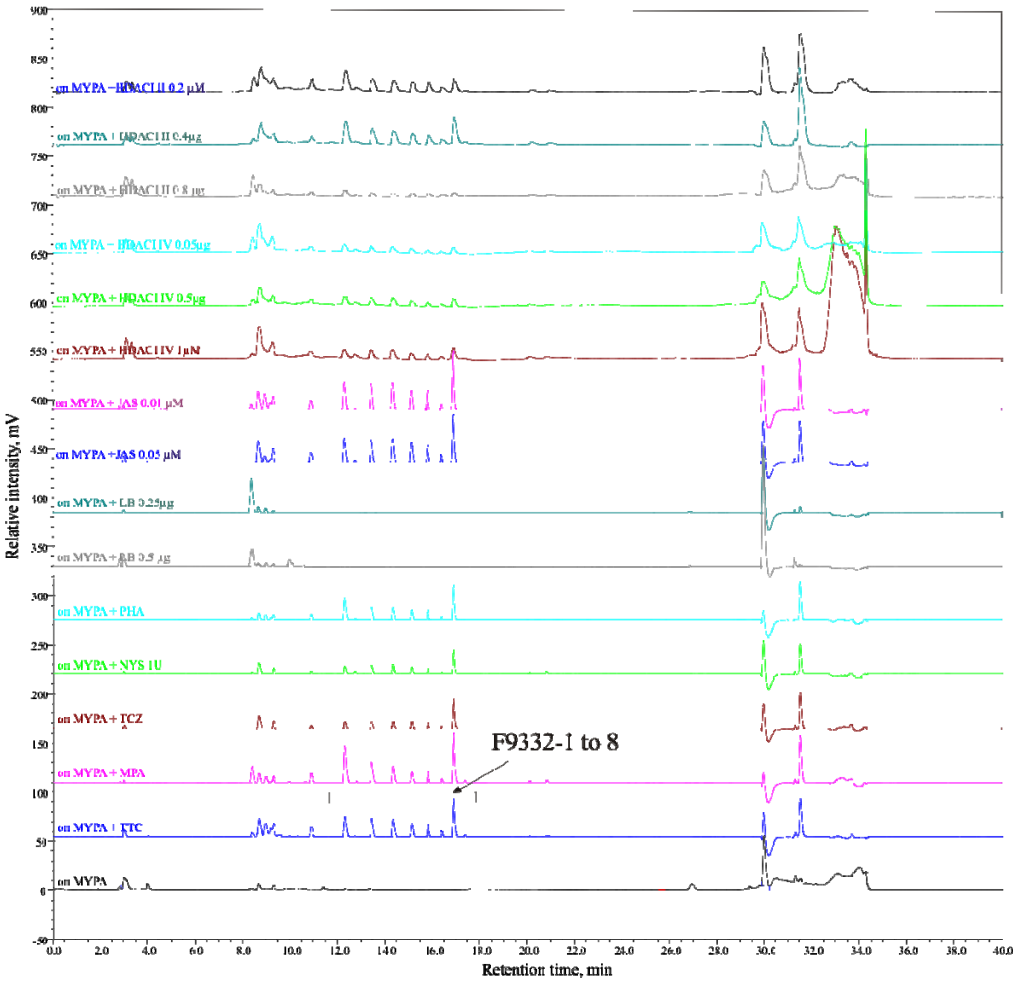


Figure 5.93: HPLC traces of extracts from *Bombardia* sp. cultured on MYPA at 20°C for 20 days with addition of different elicitors.

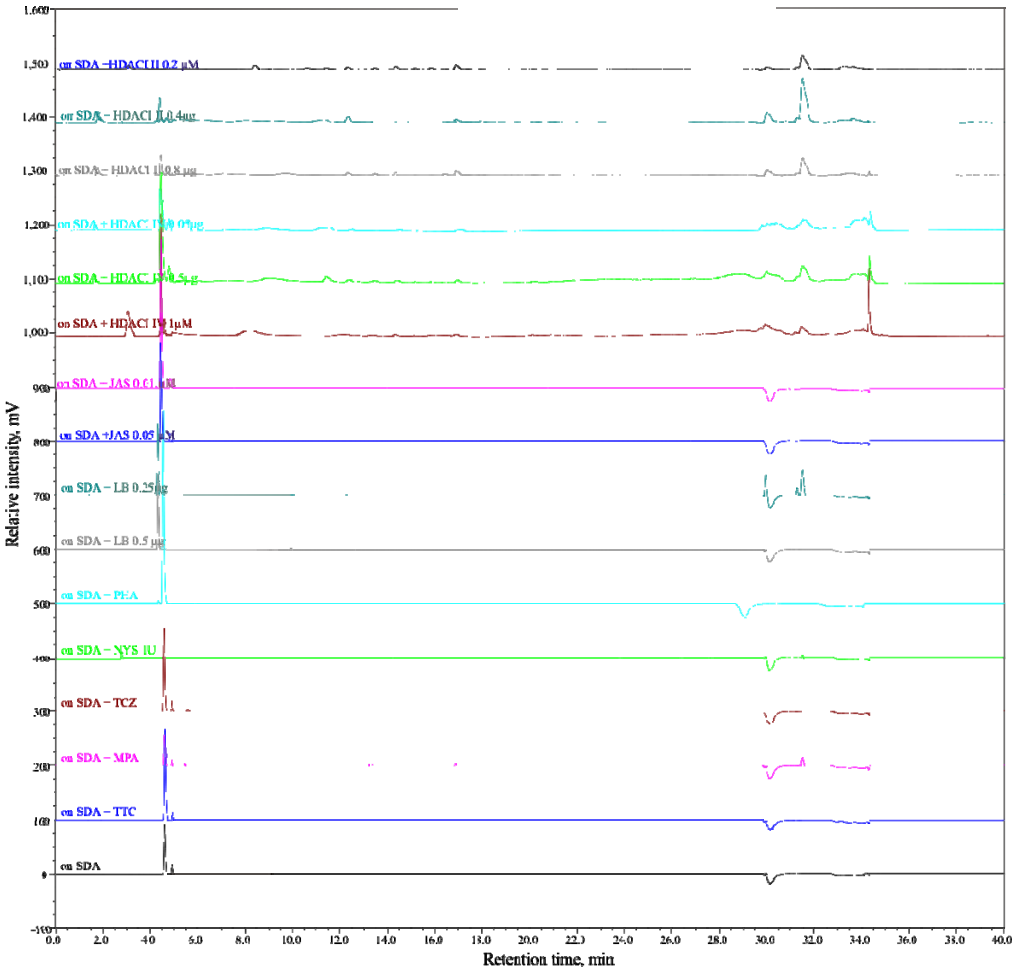


Figure 5.94: HPLC traces of extracts from *Bombardia* sp. cultured on SDA at 20°C for 20 days with addition of different elicitors.

The structural elucidation of the compounds produced by *Bombardia* sp. is discussed in **Section 5.14**.

5.15 STRUCTURAL ELUCIDATION OF THE COMPOUNDS PRODUCED BY *Bombardia* sp.

5.15.1 Extract F9330

The crude extract (F9330) from MYPA was first analysed on a reverseed-phase C_{18} column using the standard gradient (see **Chapter 2; section 2.7.3**) and indicated the presence of three to four compounds appearing at R_t 8.0-10.0 min as shown in **Figure 5.95**.

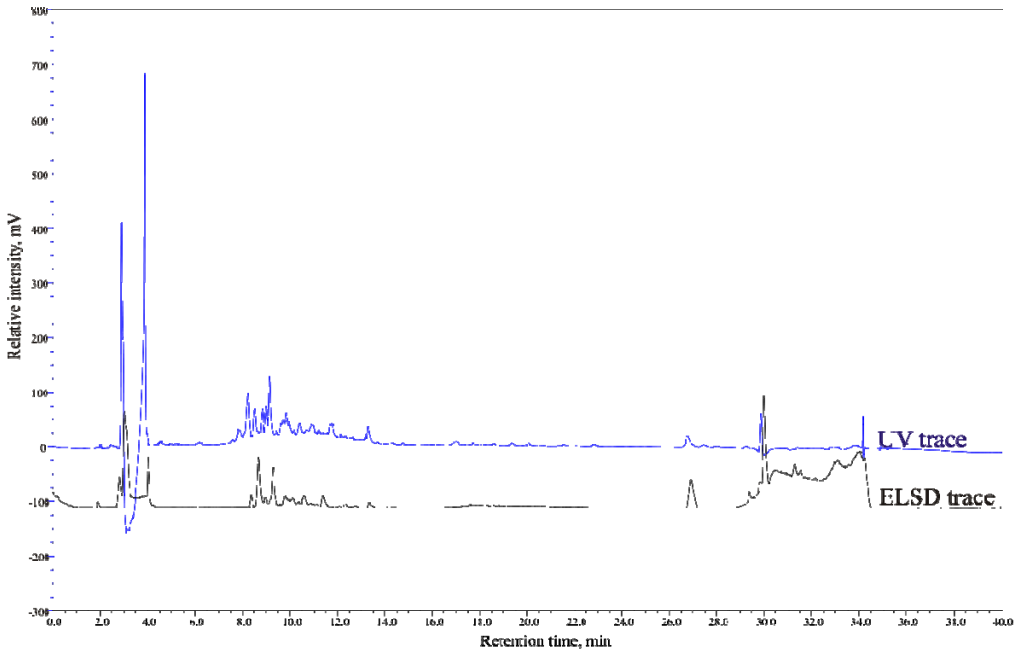


Figure 5.95: HPLC chromatogram of extract F9330 showing overlay of ELSD detection (black) and the UV detection (blue).

The separation of peaks was not adequate so the sample was re-analysed using a modified gradient system (see **Chapter 2; Section 2.8.1.1**). The HPLC chromatogram revealed the presence of four separated peaks which were

designated F9330-A1 to A4 and which eluted over the period R_t 8.5 – 11.0 min (see **Figure 5.96**).

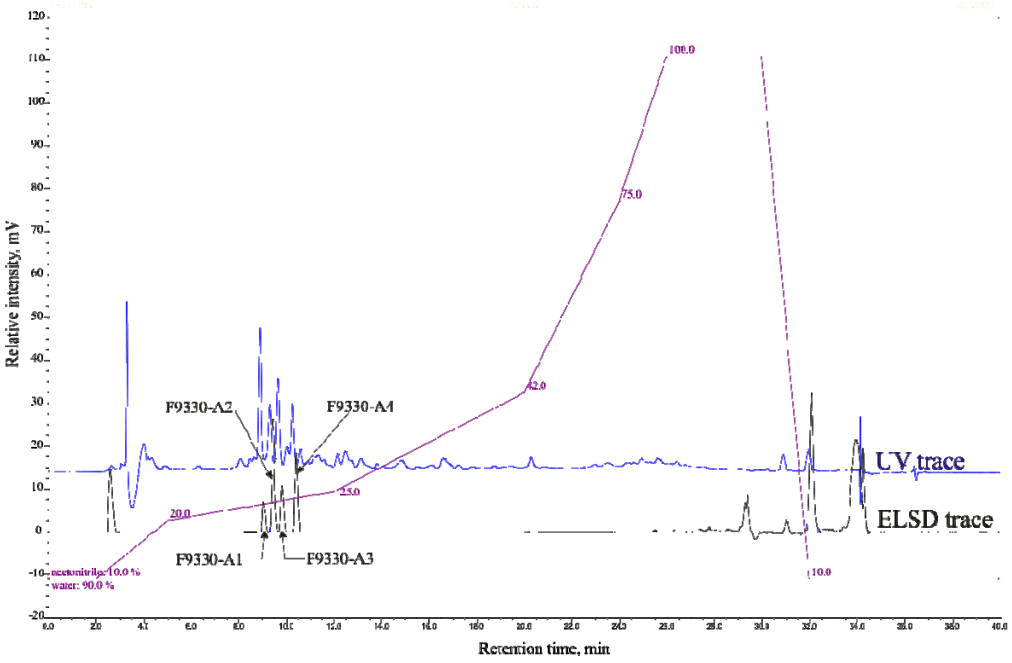


Figure 5.96: HPLC chromatogram of extract F9330 showing overlay of ELSD detection (black) and the UV detection (blue) with a modified gradient system (purple) number indicated percentage of acetronitrile run in solvent system.

5.15.1.1 Peak F9330-A1

A search of the UV profile of peak F9330-A1 in the HPLC-UV/R_t library database resulted in a good match with cycloaspeptide (**Figure 5.97**)

	Name	Match	Ret.Time[mi	Library	ID	#Extrema
1	Cycloaspeptide	963.69	15.984	Known active	A-8	3
2	excelsion	961.69	13.214	Known active	A-131	1
3	T988A	957.87	14.999	Known active	A-84	1
4	Citrinin-hydrate (?)	957.68	14.170	Known active	A-63	3
5	Auranticin B	948.46	15.899	Known active	A-158	1
6	N-Acetyl-tyramine	945.26	16.036	Known active	A	2
7	chaetoglobosin R	944.08	13.870	Known active	A-102	1
8	chaetoglobosin Q	943.07	14.309	Known active	A-101	1
9	altenusin	941.57	12.998	Known active	A-161	2
10	Psychrophilin A	941.25	16.215	Known active	A-10	3
11	(5S,5R) 5-hydroxylasiodiplodin	939.57	12.599	Known active	A-401	3
12	Cycloaspeptide	935.57	15.962	Known active	A-2	2
13	T988C	927.26	14.054	Known active	A-116	2
14	Psychrophilin	926.04	15.075	Known active	A-6	4

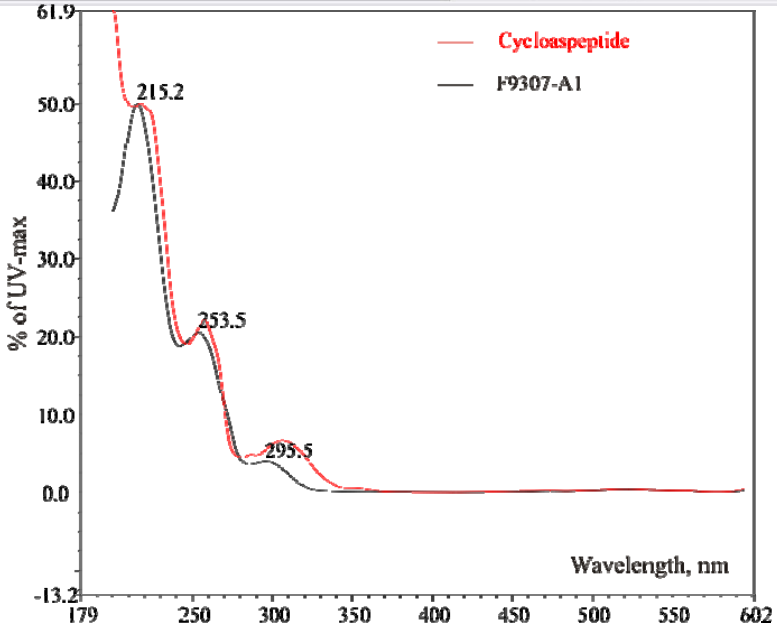


Figure 5.97: UV spectrum of peak F9330-A1 (black) and cycloaspeptide (red) with a search in the HPLC-UV/R_t library database.

The low yield of the F9330 extract was not sufficient for capNMR analysis of F9330-A1 and further culturing will be necessary to confirm the structure using NMR spectroscopy.

5.15.1.2 Peak F9330-A2

The ESIMS data for this compound (**Figure 5.98**) was of low intensity and dominated by multiple ions. No sensible conclusion could be drawn as to a likely molecular mass for F9330-A2 and therefore a possible identity.

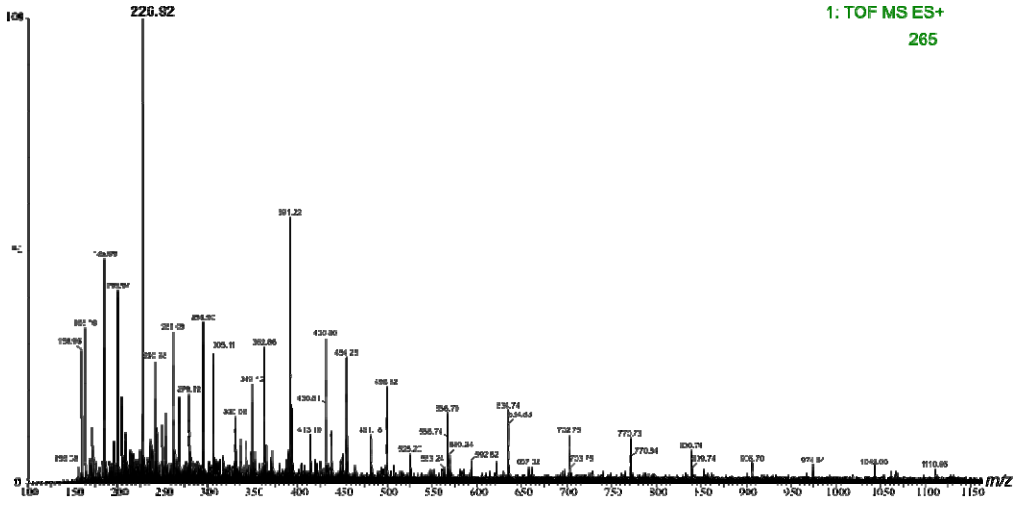


Figure 5.98: ESIMS spectrum of compound F9330-A2

However, the NMR data obtained using the CapNMR probe was adequate for interpretation. From the acquired ^{1}H NMR, HSQC and COSY NMR data for F9330-2 (see **Figures 5.99 - 5.101**) several structural features could be recognized: two doublet methyls, three methylenes, four methines and what could be assigned as either a 1,4-disubstituted, or a 1,2,3,4-tetrasubstituted benzene.

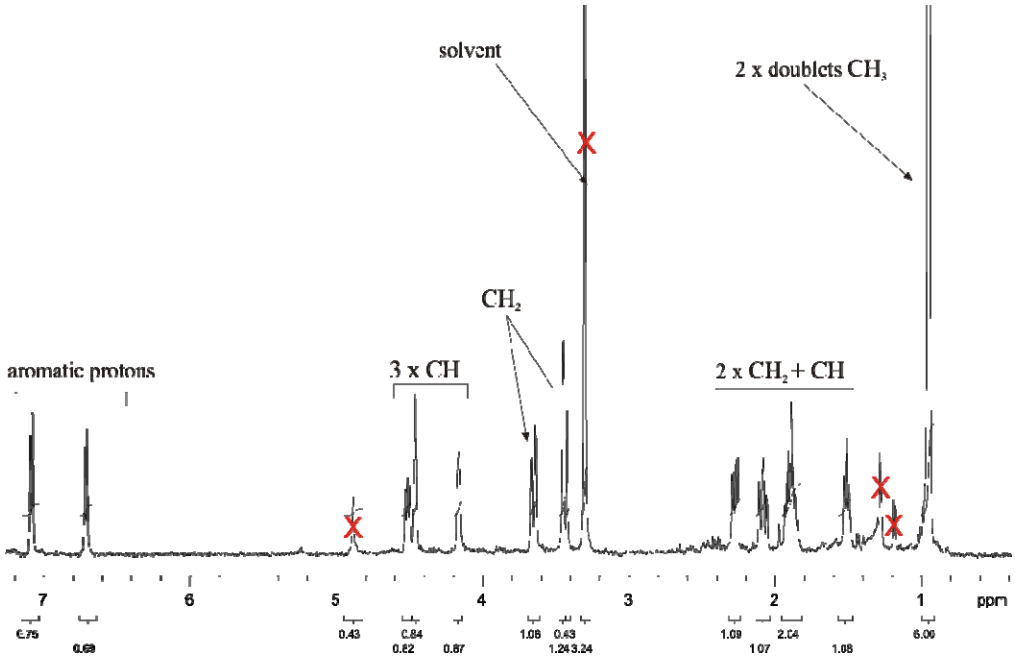


Figure 5.99: ^{1}H NMR spectrum of F9330-A2 with intregral values; 20 µg in 5.5 µL of CD_3OD, recorded at 500 MHz, 180 sec.

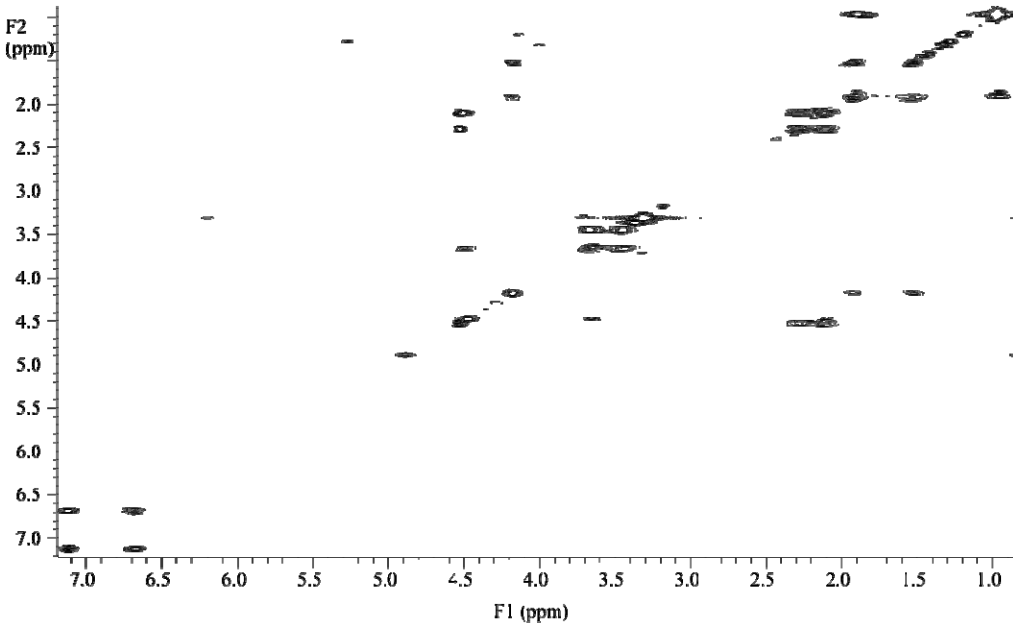


Figure 5.100: COSY NMR spectrum of F9330-A2 in CD_3OD

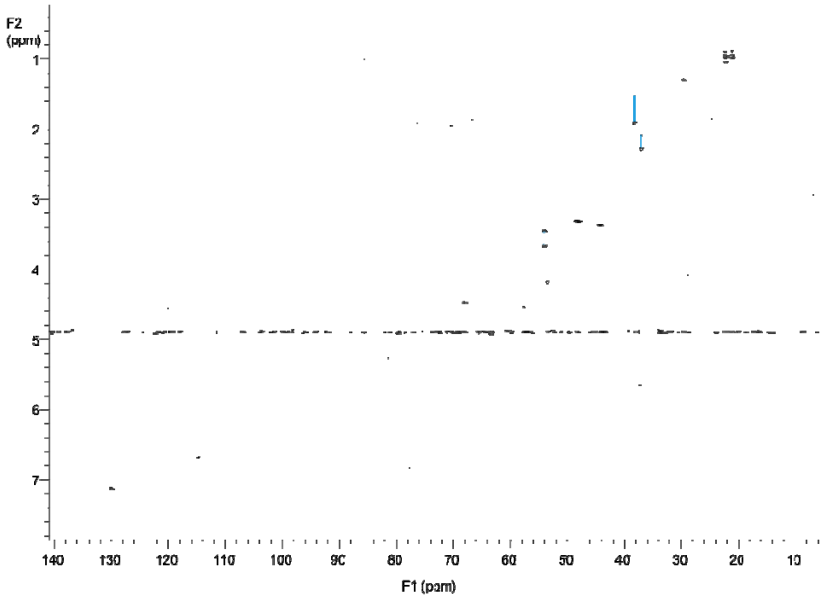


Figure 5.101: HSQC NMR spectrum of F9330-A2 in CD_3OD (blue lines represent CH_2 group).

However, when the integral values were examined a 1,4-disubstituted aromatic system seemed unlikely (should have integral values of ~2), and even a 1,2,3,4-tetrasubstituted aromatic was also a little doubtful as the integral values for the aromatic protons (δ_H 6.7 and 7.15) were less than 1H each.

It was possible to unambiguously delineate a leucine substructure from the ^{1}H/COSY NMR data (see **Figure 5.102**).

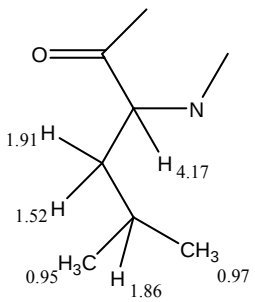
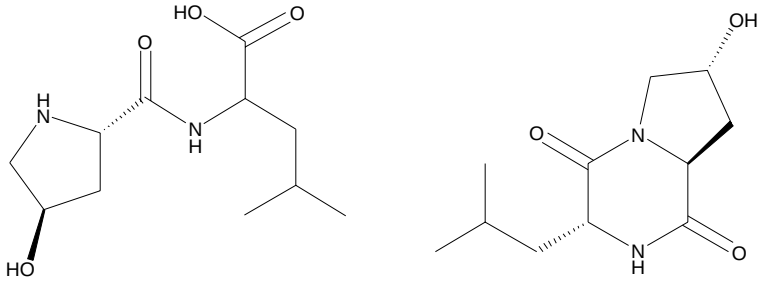


Figure 5.102: Leucine

A search was then undertaken in AntiMarin with the following search profile: two doublet methyls, three methylenes and four methines. This returned just 21 matches. Of these 9 contained a leucyl substructure. The data that had been obtained matched quite well with either Leu-4-hydroxy-Pro dipeptide (2 examples), or cyclo[(4-hydroxyprolinyl)-leucine] (6 examples) (see **Figure 5.103**).



a) Leu-4-hydroxy-Pro dipeptide **b)** cyclo[(4-hydroxyprolinyl)-leucine]

Figure 5.103: a) Leu-4-hydroxy-Pro dipeptide; **b)** cyclo[(4-hydroxyprolinyl)-leucine].

These two possibilities differ by 18 amu. The molecular mass of the dipeptide would be 244 amu and that for the cyclo[(4-hydroxyprolinyl)-leucine] would be 226 amu. Reexamination of the MS data showed an ion at *m/z* 226.92 (see **Figure 5.98**) and no distinctive ion at *m/z* 244. On this basis F9330-A2 was tentatively assigned as cyclo[(4-hydroxyprolinyl)-leucine]. The assignment of the NMR structural data for this tentative structure for F9330-A2 is shown in **Figure 5.104** and was based on ^{1}H NMR, HSQC and HMBC NMR data. The HMBC NMR data is included in **Figure 5.105**.

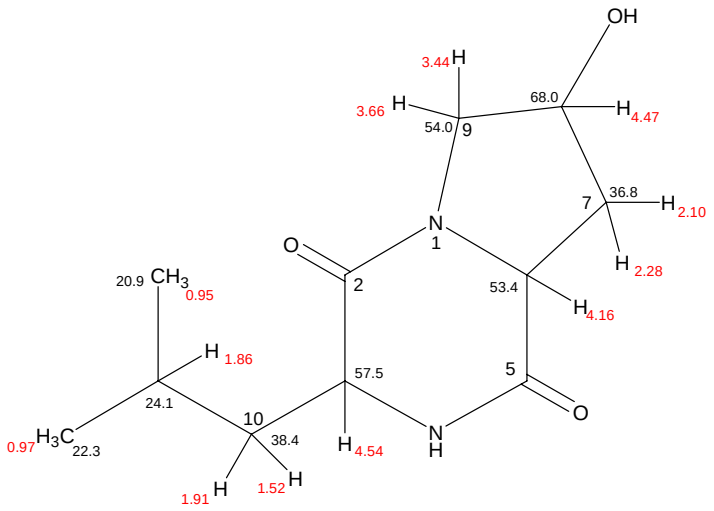


Figure 5.104: Structure of F9330-2

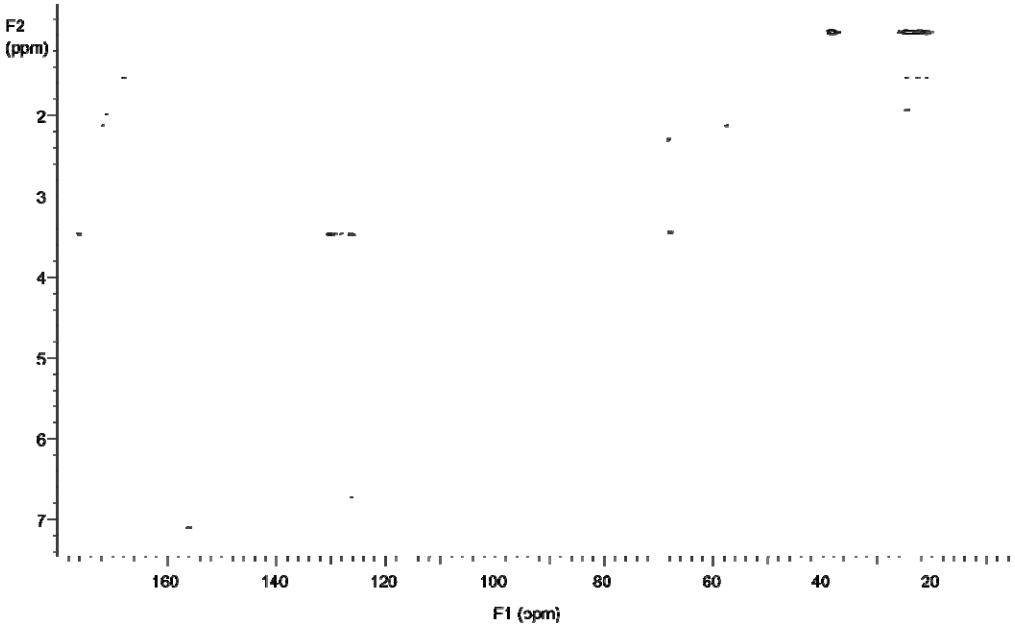


Figure 5.105: HMBC NMR spectrum of F9330-A2 in CD_3OD.

Both the *cis*- and *trans*- isomers of cyclo[L-(4-hydroxyprolinyl)-L-leucine] have been previously isolated (Cronan *et al.* 1998 and Shigemori *et al.* 1998). The ^1H NMR data reported by Shigemori had been acquired in $CDCl_3$ while

the data from Cronan and F9330-A2 had been acquired in CD_3OD. Solvent differences have a greater effect on ^1H NMR data than ^{13}C NMR data. **Table 5.19** lists the published data (in CD_3OD and $CDCl_3$) for the *cis*- and *trans*-isomers along with the comparable data for F9330-A2 in CD_3OD.

Table 5.19: ^1H NMR and ^{13}C NMR data of F9330-A1 and *cis*- and *trans*-isomers of cyclo-4-hydroxyprolinyl-leucine

Position	run in CD_3OD (500 MHz)		^1H NMR in CD_3OD, ^{13}C NMR in D_2O (300 MHz)				run in $CDCl_3$ (500 MHz)	
	F9330-A2		cyclo[L-*trans*-4-hydroxyprolinyl-L-leucine]		cyclo[L-*cis*-4-hydroxyprolinyl-D-leucine]		cyclo[D-*cis*-4-hydroxyprolinyl-L-leucine]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		167.7		169.0		169.4		167.7
3	4.18	53.4	4.15	55.2	3.84	57	3.95	56.2
4			7.56				7.56	
5		171.9		173.0		171.2		169.8
6	4.52	57.5	4.51	58.7	4.33	57.6	4.16	56.1
7a	2.10	36.8	2.08	37.7	2.24	37.7	2.45	36.8
7b	2.28		2.27		2.47		2.45	
8	4.47	68.0	4.45	68.9	4.40	68.9	4.45	68.1
9a	3.44	54.0	3.42	54.6	3.43	54.3	3.34	54.0
9b	3.66		3.65		3.65		3.95	
10	1.52	38.4	1.50	39.4	1.61	43.4	1.63	42.2
10b	1.91		1.90		1.61		1.63	
11	1.86	24.1	1.88	25.8	1.74	25.6	1.75	24.6
12	0.95	20.9	0.95	22.2	0.95	22	0.95	21.5
13	0.97	22.3	0.98	23.3	0.98	23.3	0.98	23.0

A careful comparison of the data, especially the ^{13}C NMR data (extracted from the HSQC and HMBC NMR spectra) suggested that F9330-A2 was the *trans* isomer. The absolute configuration as L/L or D/D could not be assigned, although the L/L is shown in **Figure 5.106**.

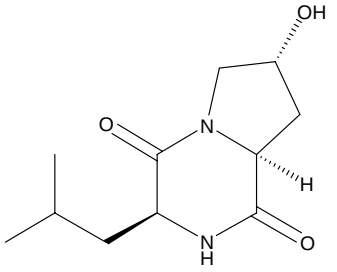
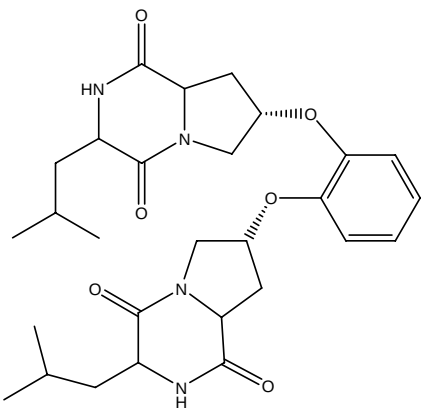
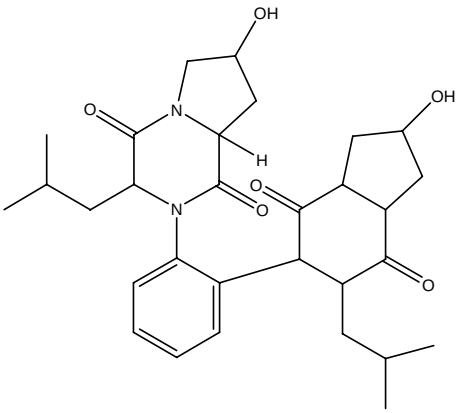


Figure 5.106: cyclo[L-*trans*-4-hydroxyprolinyl-L-leucine]

Unfortunately, this can only be a tentative assignment even though a potential structure had been obtained. This is because the presence of aromatic protons in this sample cannot be considered spurious. I am grateful to the Internal Examiner, Dr. Sylvia Urban, for pointing out this anomaly. She has suggested some alternative, more complex structures that could account for these aromatic resonance (see **Figure 5.107**). Until more definitive NMR and ESIMS can be obtained the actual structure for F9330-A2 remains unassigned.



Structure 1: MW= 526 amu



Structure 2: MW= 524 amu

Figure 5.107: Suggested possible structures for fraction F9330-A2

5.15.1.3 Peak F9330-A3

A search of UV profile of peak F9330-A3 in the HPLC-UV/R_t library database resulted in a close match with pseurotin A3 (**Figure 5.108**).

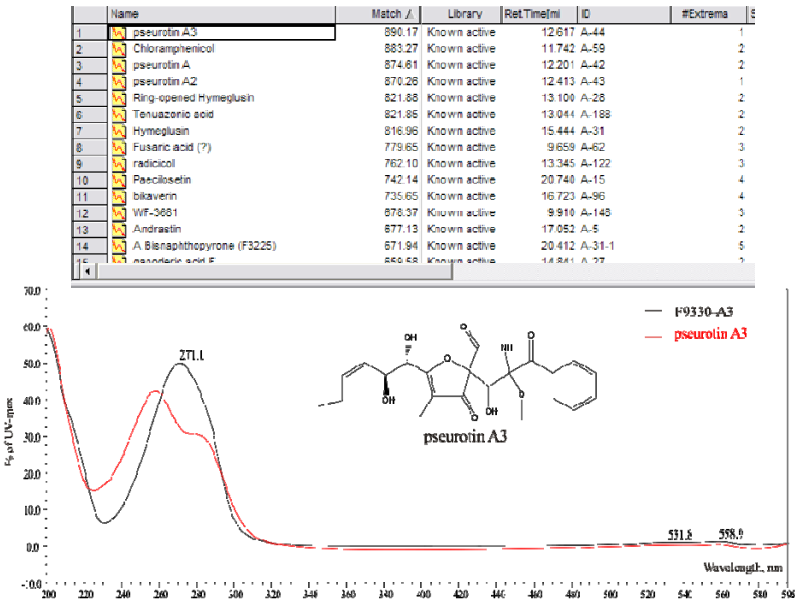


Figure 5.108: UV spectrum of peak F9330-A3 (black) and cycloaspeptide (red) with a search in the HPLC-UV/R_t library database.

The ^{1}H NMR spectrum revealed the fraction F9330-A3 contained impurities and could be a mixture of compounds. A greater amount of F9330-A3 is required for structural elucidation.

5.15.1.4 Peak F9330-A4

The extracted UV spectrum of peak F9330-A4, given below in **Figure 5.109** showed no matches in the HPLC-UV/R_t library database.

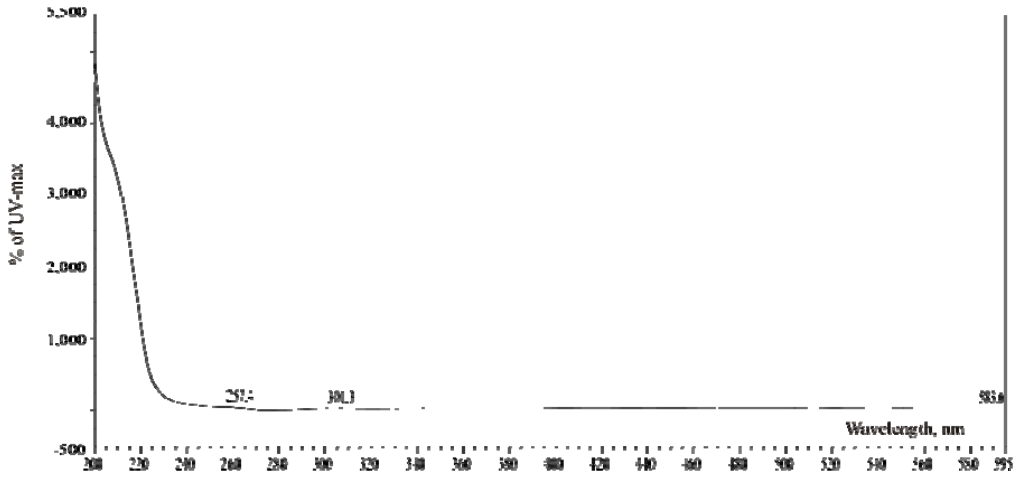


Figure 5.109: Extracted UV profile for compound F9330-A4.

As was observed for F9330-A2 the ESIMS data for this compound (**Figure 5.110**) was inconclusive. Again it was of low intensity and dominated by multiple ions. No sensible conclusion could be drawn as to a likely molecular mass for F9330-A4.

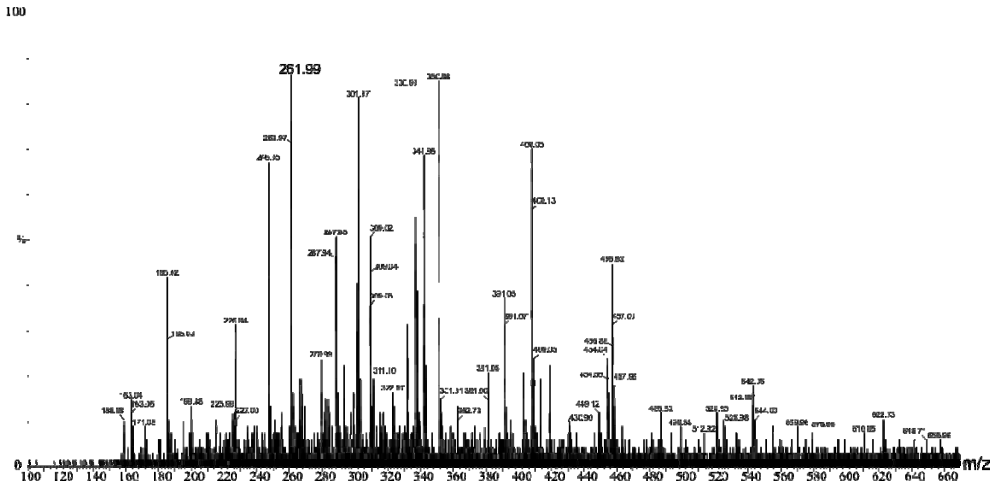


Figure 5.110: ESIMS spectrum of compound F9330-A4

As for F9330-A2, the CapProbe ^{1}H NMR spectrum of F9330-4 was instructive in revealing the presence of three methylenes, three methines and one monosubstituted benzene ring as shown in **Figure 5.111**

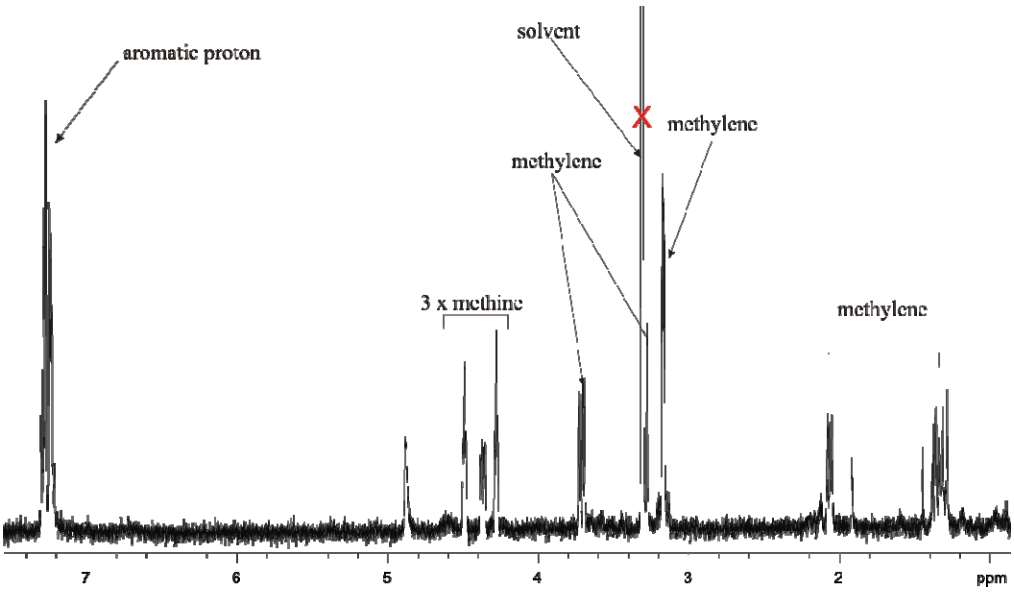


Figure 5.111: ^{1}H NMR spectrum of F9330-A4; 15 µg in 5.5 µL of CD_3OD, recorded at 500 MHz, 180 sec.

A search of the AntiMarin database using the following search profile: zero methyl groups, three methylenes, three methines, one monosubstituted benzene (**Figure 5.112a**) returned eight matches of which seven were diketopiperazines (**Figure 5.112b**). Of that seven, six were cyclo[4-hydroxyl-prolynyl)-phenylalanyl] isomers. The molecular mass of that compound was 260.288 amu. Reexamination of the ESIMS confirmed that there was an ion at *m/z* 261 amu (see **Figure 5.110**).

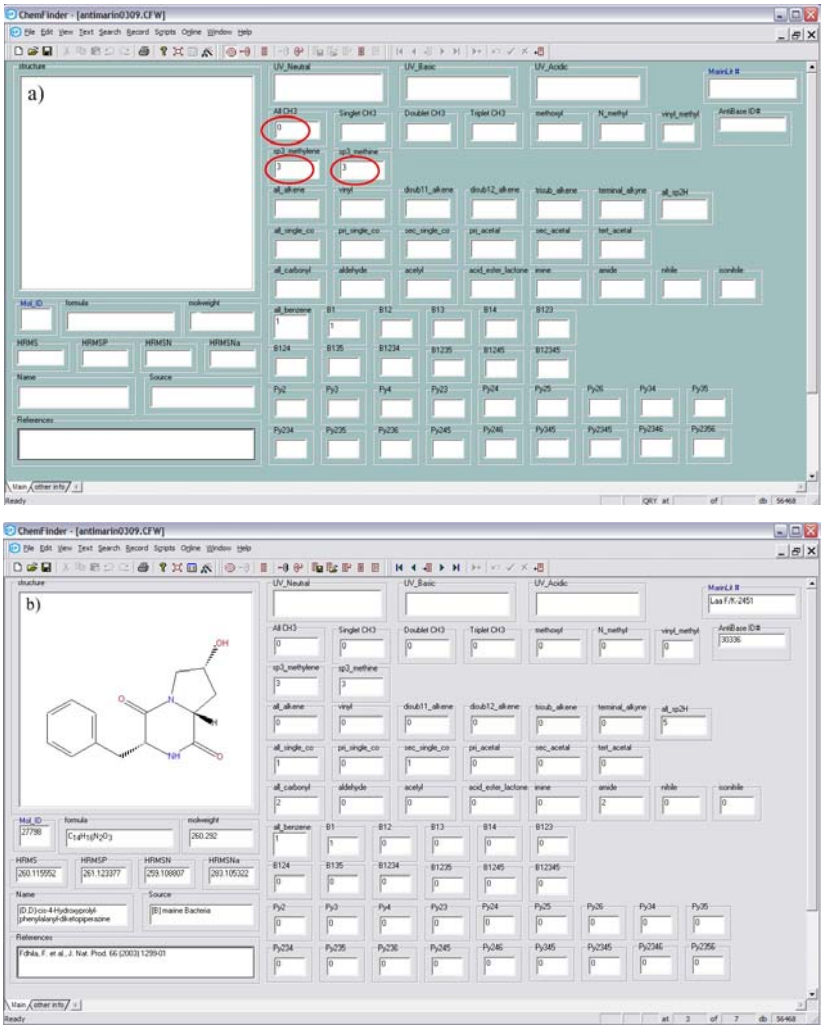


Figure 5.112: **a)** AntiMarin search profile for F9330-A4; **b)** AntiMarin search result for F9330-A4.

^{1}H and ^{13}C NMR data previously reported for cyclo[(4-hydroxylprolyl)-phenylalanyl] (Fdhila, 2003; Furtado, 2005) suggested that the data were consistent with that obtained for cyclo[D-*cis*-4-hydroxylprolyl-D-phenylalanyl] (**Figure 5.113**).

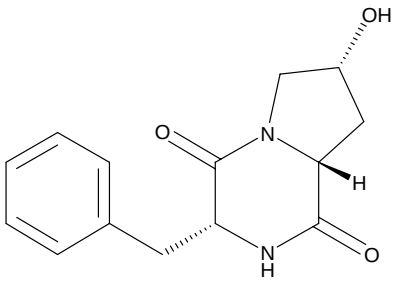


Figure 5.113: cyclo[D-*cis*-4-hydroxylprolyl-D-phenylalanyl]

5.15.2 Extract F9332

The crude extract F9332 was obtained from MYPA + MPA culture. HPLC analysis revealed the presence of eight peaks appearing across the region R_t 12-18 min as shown in **Figure 5.114**.

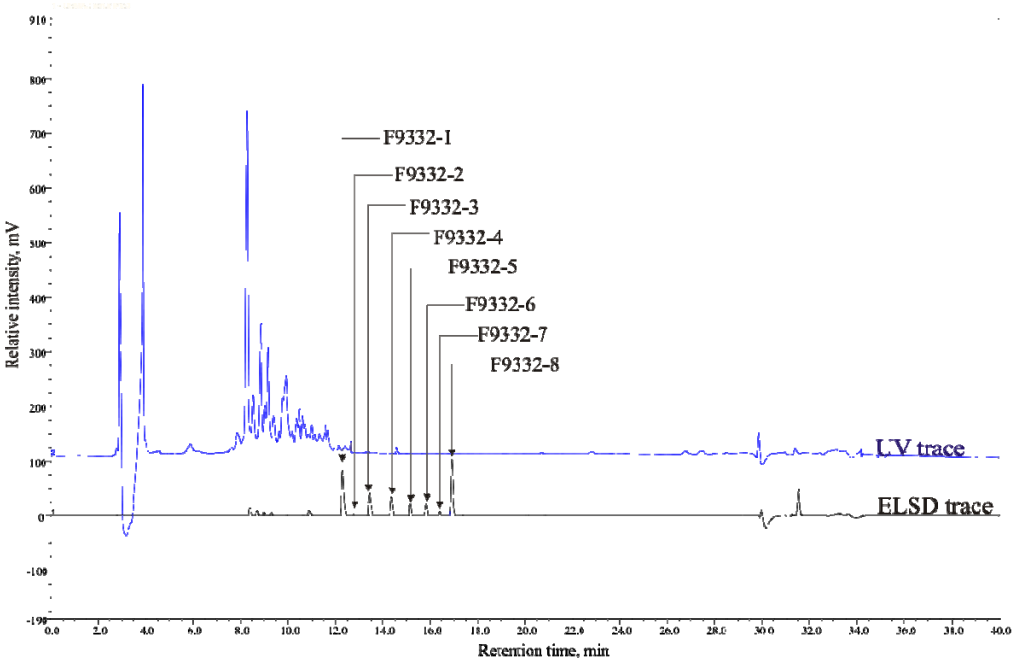


Figure 5.114: HPLC chromatogram of extract F9332 showing overlay of ELSD detection (black) and the UV detection (blue).

An interesting preliminary observation of fraction F9332-1 to F9332-8 was that none of these compounds displayed a UV chromophore with one exception in the UV trace. (see **Figure 5.115**).

After fractionation of 700 mg of the extract F9332 followed by HPLC microtitre plate collection, the ^{1}H NMR spectra of each of the eight fractions were acquired using the CapNMR probe. The ^{1}H NMR spectrum of fraction F9332-8 is shown in **Figure 5.115**. The ^{1}H NMR spectra of fractions F9332-1 and F9332-4 were identical to that of F9332-8 (**Figure 5.116**). This suggested that F9332-1 to F9332-8 were based on a common repeating subunit.

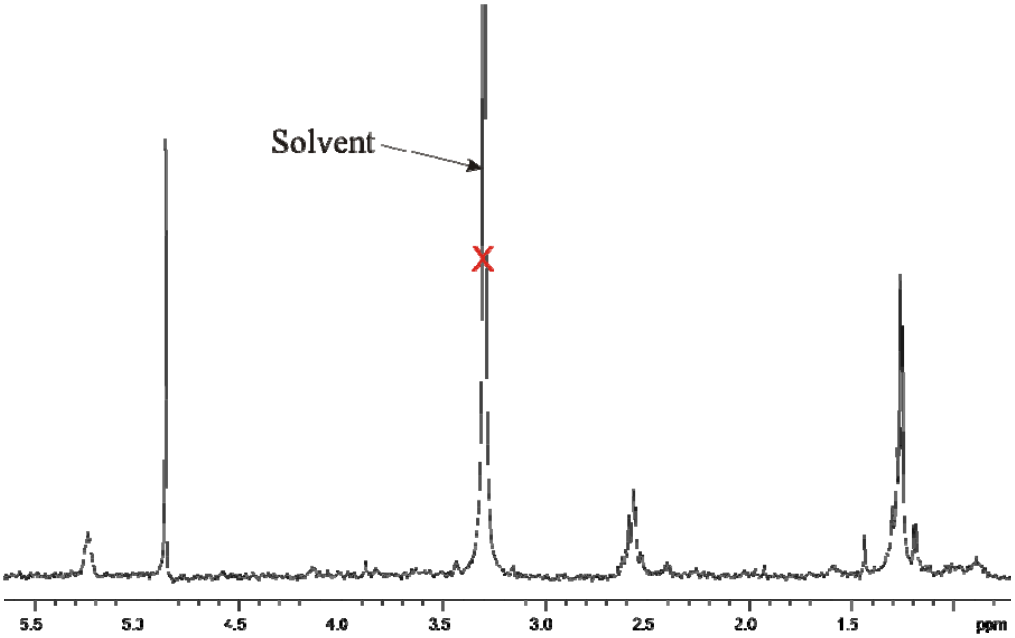


Figure 5.115: ^{1}H NMR spectrum of F9332-8; 29 µg in 5.5 µL of CD_3OD, recorded at 500 MHz, 240 sec

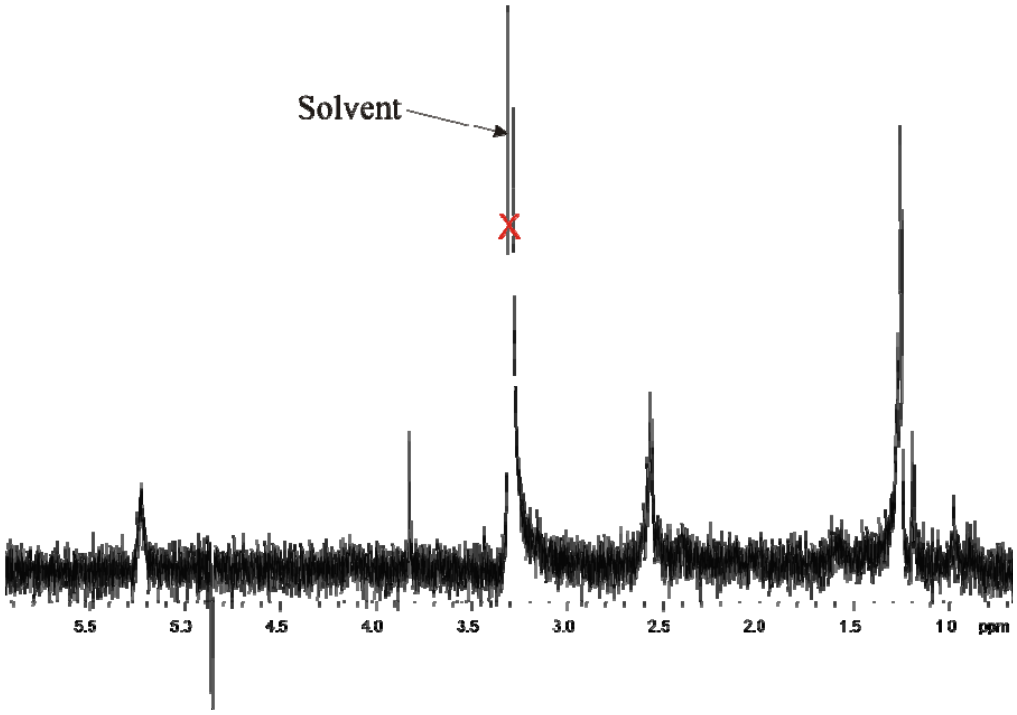


Figure 5.116: ^{1}H NMR spectrum of F9332-4; 10 µg in 5.5 µL of CD_3OD, 500 recorded at MHz, 240 sec.

LCMS (**see method in Chapter 2; Section 2.8.3**) was used to identify the molecular masses and relationships among the fractions (**Figure 117**). The positive ESIMS of fraction F9332-1 to 8 showed that each differed from the next by 86 amu (**Figure 5.118**).

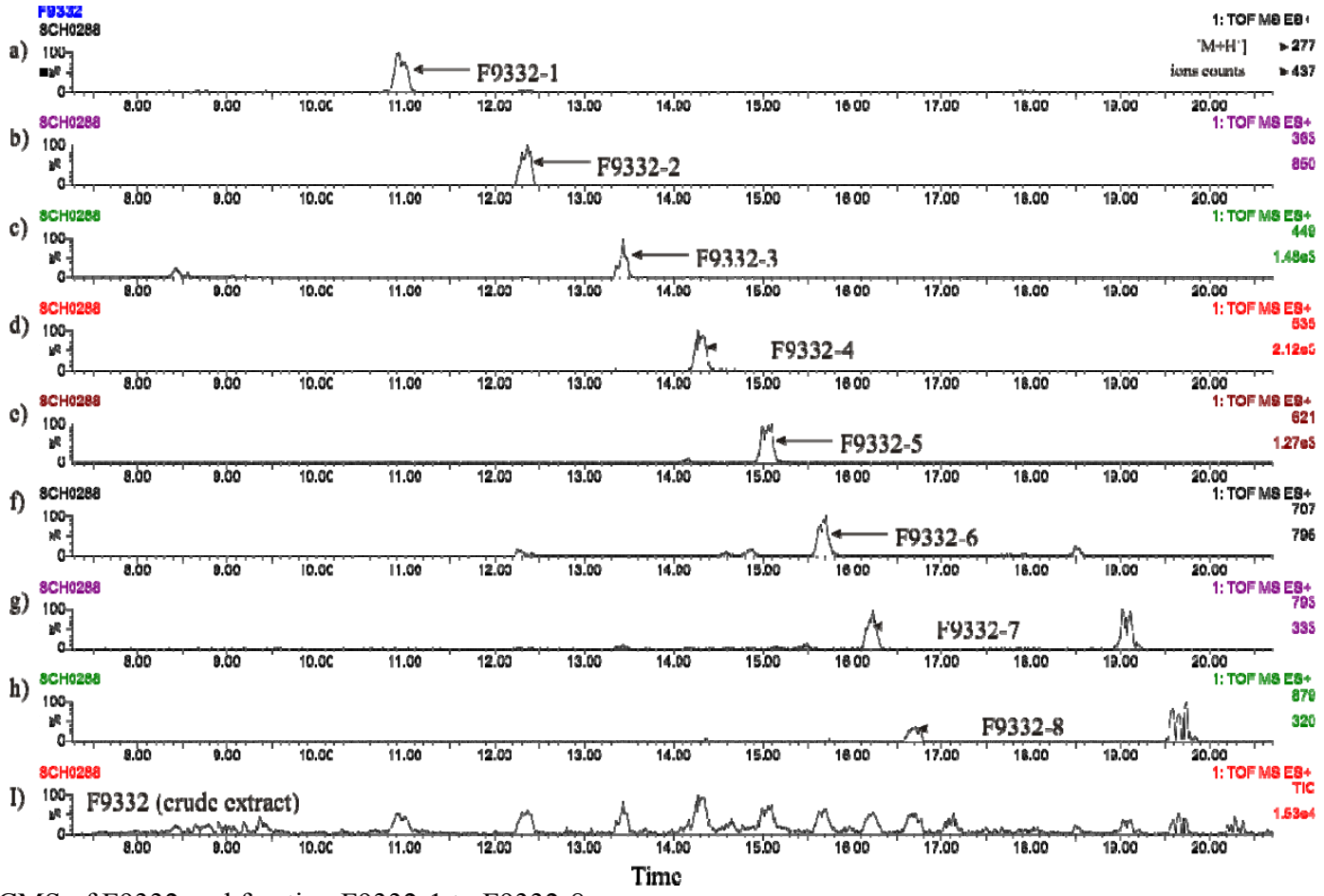


Figure 5.117: LCMS of F9332 and fraction F9332-1 to F9332-8.

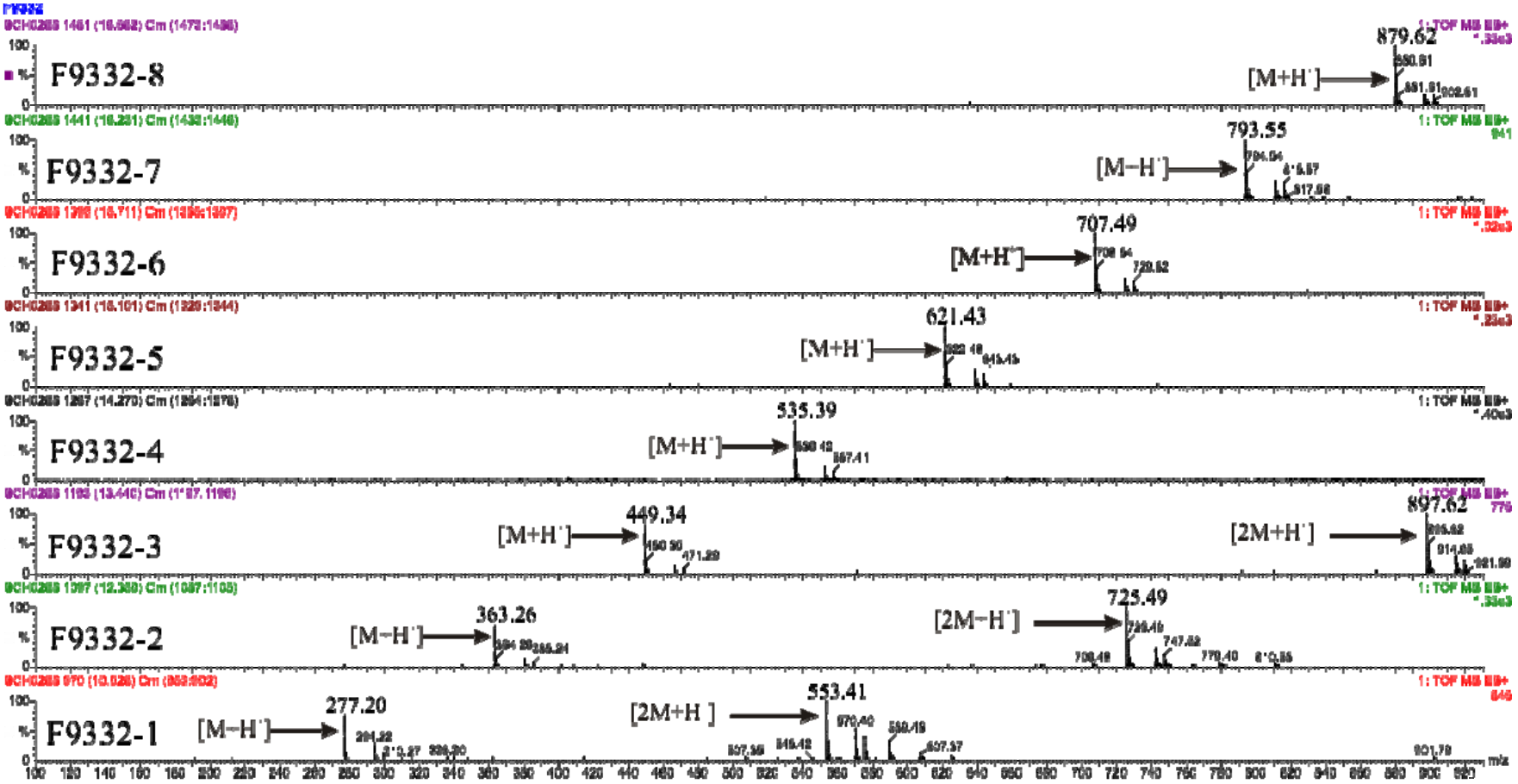
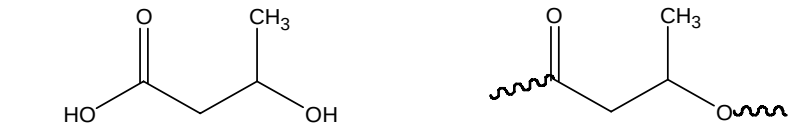


Figure 5.118: A comparison of ESIMS of fraction F9332-1 to 8.

The ^{1}H NMR and LCMS data allowed the assignment of the repeating substructure unit as 3-hydroxybutanoic acid (**Figures 5.119a** and **5.119b**).



a) 3-hydroxybutanoic acid MW= 104 b) subunit MW = 86 amu

Figure 5.119: 3-hydroxybutanoic acid and the repeating subunit.

From ESIMS the molecular mass of peak F9332-1 was 277 amu ([M+H^{+}]) corresponding to the esterification of three molecules of 3-hydroxybutanoic acid (**Figure 5.120**).

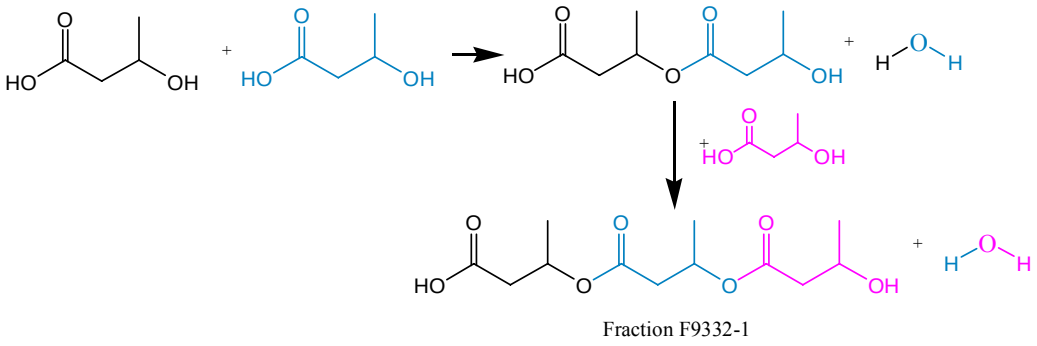
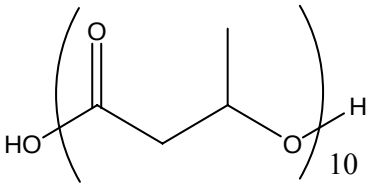


Figure 5.120: Esterification of 3-hydroxybutanoic acid

With an increase of 86 amu from fraction to fraction, structures could be suggested for the series culminating in the structure of F9332-8 as a polymer of 10 molecules of 3-hydroxybutanoic acid ($C_{40}H_{62}O_{21}$); poly-3-hydroxybutyrate **(Figure 5.121**). This compound had previously been found

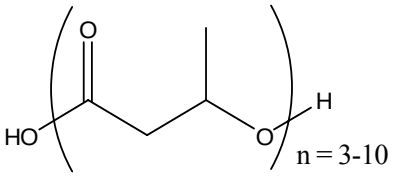
in various bacteria such as *Alcaligenes eutrophus* or *Bacillus megaterium* (López *et al*., 1998; Rueping *et al*., 2001; and Fukui *et al*., 2009).



Fraction F9332-8

Figure 5.121: Structure of Fraction F9332-8

The calculated molecular weight of fraction F9332-8 is 878 amu in agreement with the observed molecular mass from ESIMS of 879 amu ([M+H^+]). Thus compounds F9332-1 to F9332-8 are poly-3-hydroxybutyrates (**Figure 5.122**).



Fraction F9332-1 to 8

Figure 5.122: Poly-3-hydroxybutyrates

Part E: *Chaetomium trigonosporum*

5.16 CULTURE CHARACTERISTIC AND MORPHOLOGY

Fungal strain 702_3@20.1 was isolated from soil collected from Tasman Bay (3% phenol plate). This culture shared the characteristic features of the ascomyceteous genus *Chaetomium*. The presence of perithecia with perithecial hairs and a *Scopulariopsis*-like conidial state (**Figure 5.123a**) was consistent with this identification. Colonies on MYPA at 20 °C showed a daily growth rate of 2.5-3.3 mm. Ascomata are produced in abundance on the culture and reached maturity within 10 days (**Figure 5.123b**).

Perithecia were ovate, ostiolate, 200-250 µm × 133-155 µm and bear setae-like hairs. Hairs were straight, 3.0-5.5 µm diameter at the base, 100-300 µm length. Asci contained 8 ascospores (**Figure 5.123c**) and are evanesence. Ascospores are triangular in front view, ellipsoidal in side view and dark brown when mature, 4.5-5.0 × 6.6-7.0 × 1.0-1.2 µm (hight × width × depth) (**Figure 5.123d**).

These very characteristic feaures were consistent with the isolate being *Chaetomium trigonosporum* (Arx *et al.*;1986 and Corlett; 1966).

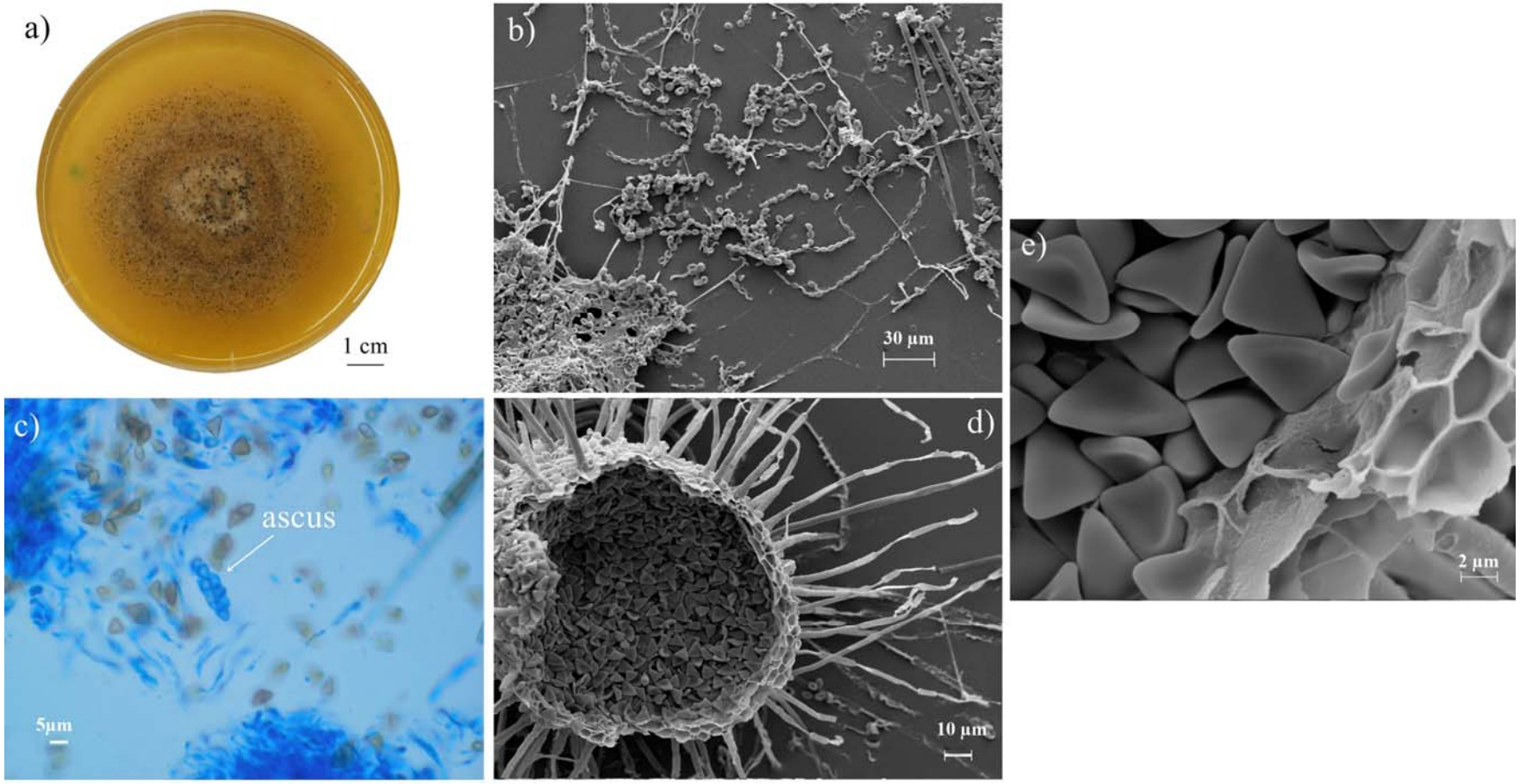


Figure 5.123: *Chaetomium trigonosporum*; **a)** 20 day culture at 25 °C on MYPA plate; **b)** SEM of conidiospores; **c)** ascus contained 8 immature ascospores; **d)** SEM of cross section of perithecium; **e)** ascospores.

5.17 EFFECT OF ELICITORS ON THE GROWTH AND THE METABOLITE PRODUCTION

5.17.1 Effect of elicitors on the growth of *C. trigonosporum*

C. trigonosporum was grown on MYPA and SDA at 20 °C for 20 days. The colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of mycelial growth in the presence of elicitors in MYPA and SDA media is shown in **Figures 5.124** and **5.125** respectively. Ascocarp production was observed and is presented in **Table 5.20**

Table 5.20: Observation of ascomata production at 20 days incubation.

Elicitors	Observation of visible ascocarps	
	MYPA	SDA
Control	++	+++
TTC	+++	++
MPA	+++	++
TCZ	++	++
NYS 1 U	++	++
PHA 0.1 U	+++	++
LB 0.5 µg	+	+
LB 0.25 µg	++	++
JAS 0.05 µM	++	++
JAS 0.01 µM	+++	++
HDACI IV 1 µM	N/O	+
HDACI IV 0.5 µM	++	++
HDACI IV 0.05 µM	++	++
HDACI II 0.8 µM	++	++
HDACI II 0.4 µM	+++	++
HDACI II 0.2 µM	+++	+++

* +++ visible ascomata covering entire colony, ++ visible ascomata abundance at the edge of the colony, + visible ascomata under dissection microscope, N/O no ascomata production.

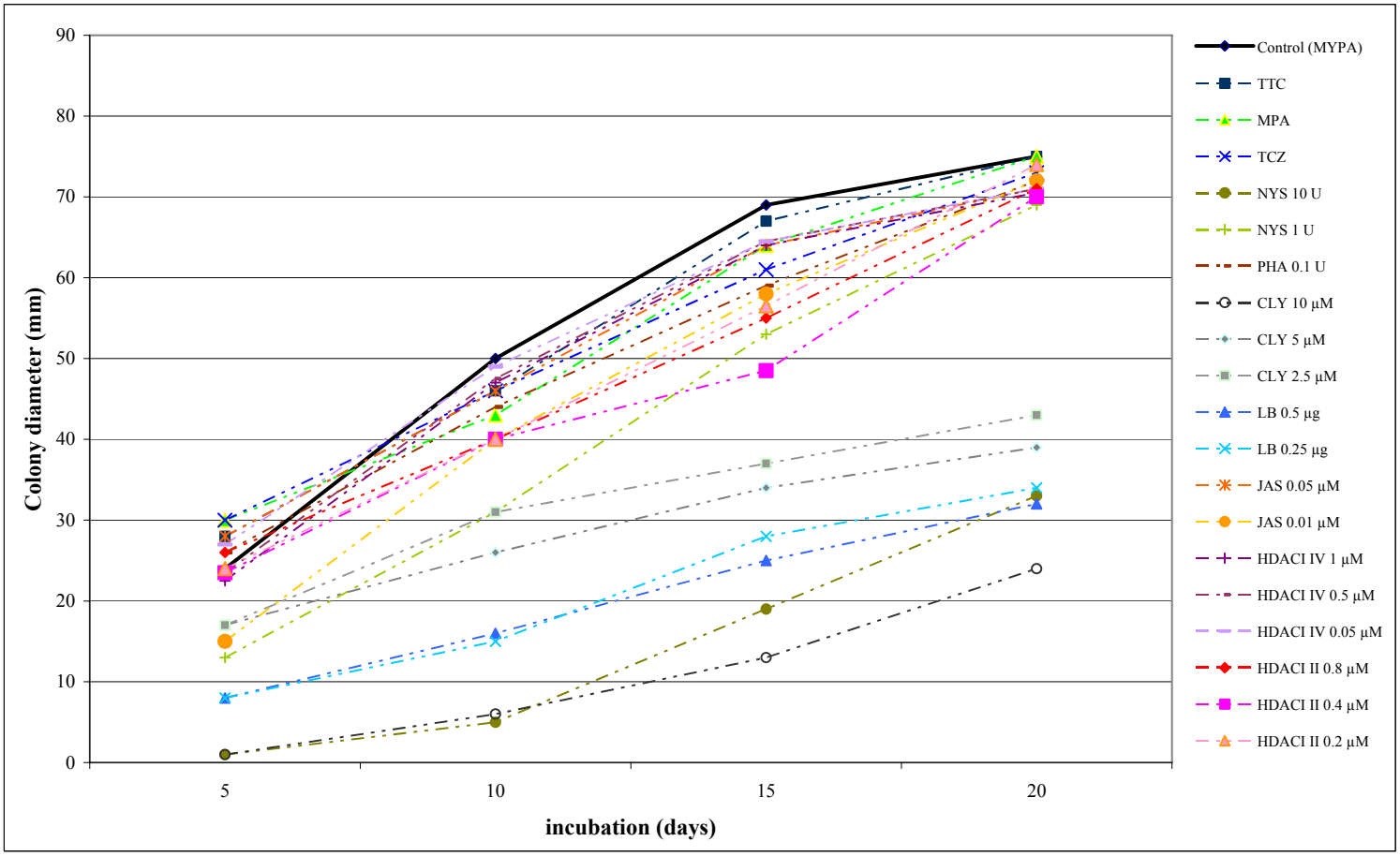


Figure5.124: Effect of elicitors on the growth of *C.trigonosporum*. on MYPA medium at 20 °C

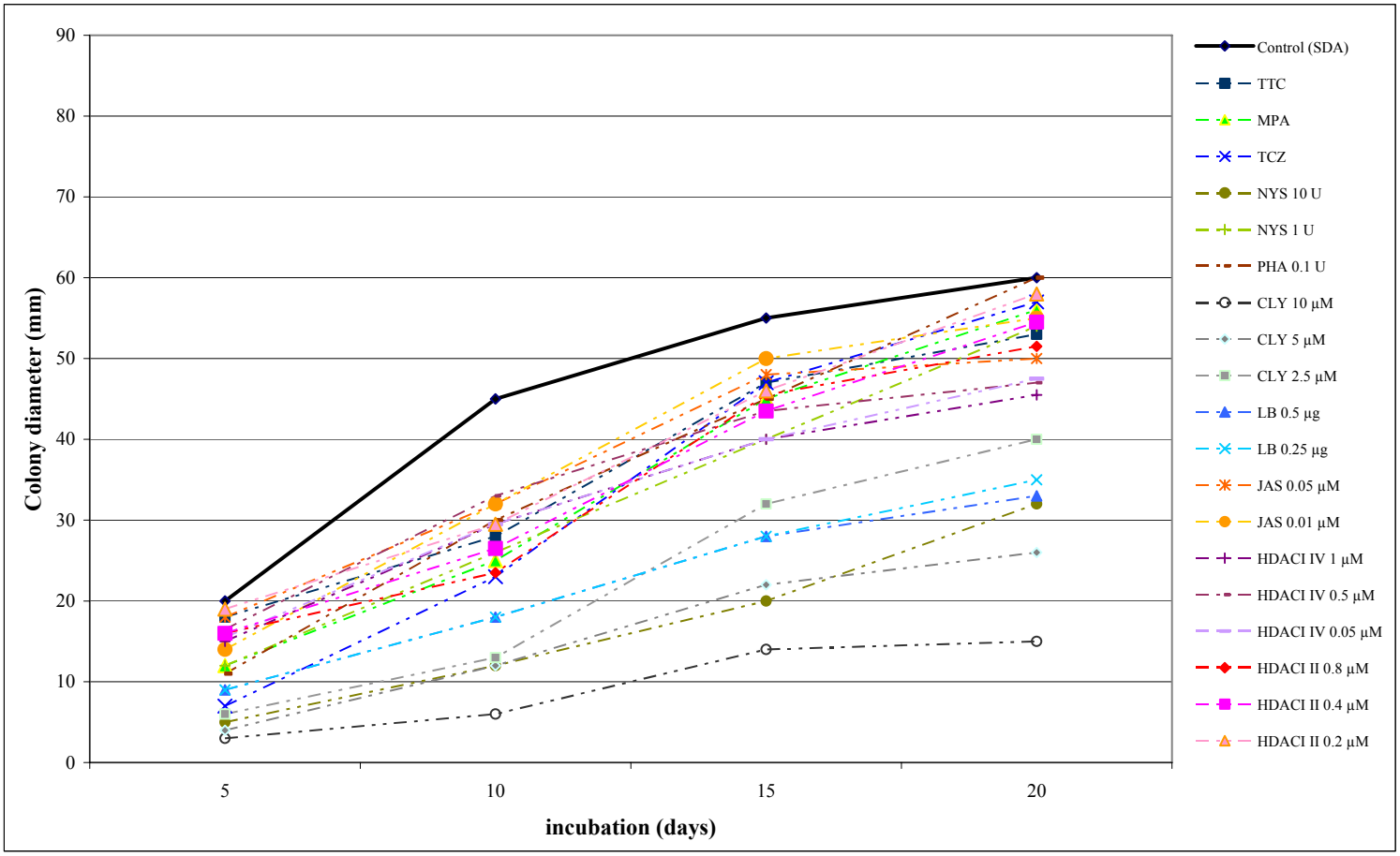


Figure5.125: Effect of elicitors on the growth of *C.trigonosporum*. on SDA medium at 20 °C

Growth of the fungus was better on MYPA as the media than SDA. No growth was observed in the presence of NYS (100 U). All other elicitors showed suppression of growth on both media. The greatest inhibition was seen in the presence of CLY (10 μM), but several elicitors showed moderate suppression; NYS (10 U), CLY (5 and 2.5 μM) and LB (0.5 and 0.25 μg). Ascocarp production was greatly effected by the presence of HDACI IV (1 μM) and LB (0.5 μg) in the media.

5.17.2 Effect of the elicitors on the cytotoxicity of the extracts and the metabolite production

5.17.2.1 Effect of elicitors on cytotoxicity of extracts

The cytotoxicity of the extracts from each treatment is shown in **Table 5.21**. The extracts from MYPA and SDA without any addition of elicitor were considered as being active against P388 cells (IC_{50} <12,500), while the rest showed no cytotoxicity.

Table 5.21: Effect of elicitors on cytotoxicity of extracts from *C. trigonosporum* on MYPA and SDA media (20 °C for 20 days).

Elicitors	Cytotoxicity on both media			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9270	11,760	F9285	11,713
TTC	F9271	>12,500	F9286	>12,500
MPA	F9272	>12,500	F9287	>12,500
TCZ	F9273	>12,500	F9288	>12,500
NYS 10 U	F9275	>12,500	F9290	>12,500
NYS 1 U	F9276	>12,500	F9291	>12,500
CLY 10 µM	F9277	>12,500	F9292	>12,500
CLY 5 µM	F9278	>12,500	F9293	>12,500
CLY 2.5 µM	F9279	>12,500	F9294	>12,500
PHA 0.1U	F9280	>12,500	F9295	>12,500
LB 0.5 µg	F9281	>12,500	F9296	>12,500
LB 0.25 µg	F9282	>12,500	F9297	>12,500
JAS 0.05 µM	F9283	>12,500	F9298	>12,500
JAS 0.01 µM	F9284	>12,500	F9299	>12,500
HDACI IV 1 µM	F9449	>12,500	F9456	>12,500
HDACI IV 0.5 µM	F9451	>12,500	F9458	>12,500
HDACI IV 0.05 µM	F9453	>12,500	F9460	>12,500
HDACI II 0.8 µM	F9542	>12,500	F9548	>12,500
HDACI II 0.4 µM	F9544	>12,500	F9550	>12,500
HDACI II 0.2 µM	F9546	>12,500	F9552	>12,500

5.17.2.2 HPLC screening of the extracts

A comparison of ELSD traces showed no differences in metabolite production on either MYPA or SDA with any elicitor (**Figures 5.126** and **5.127**)

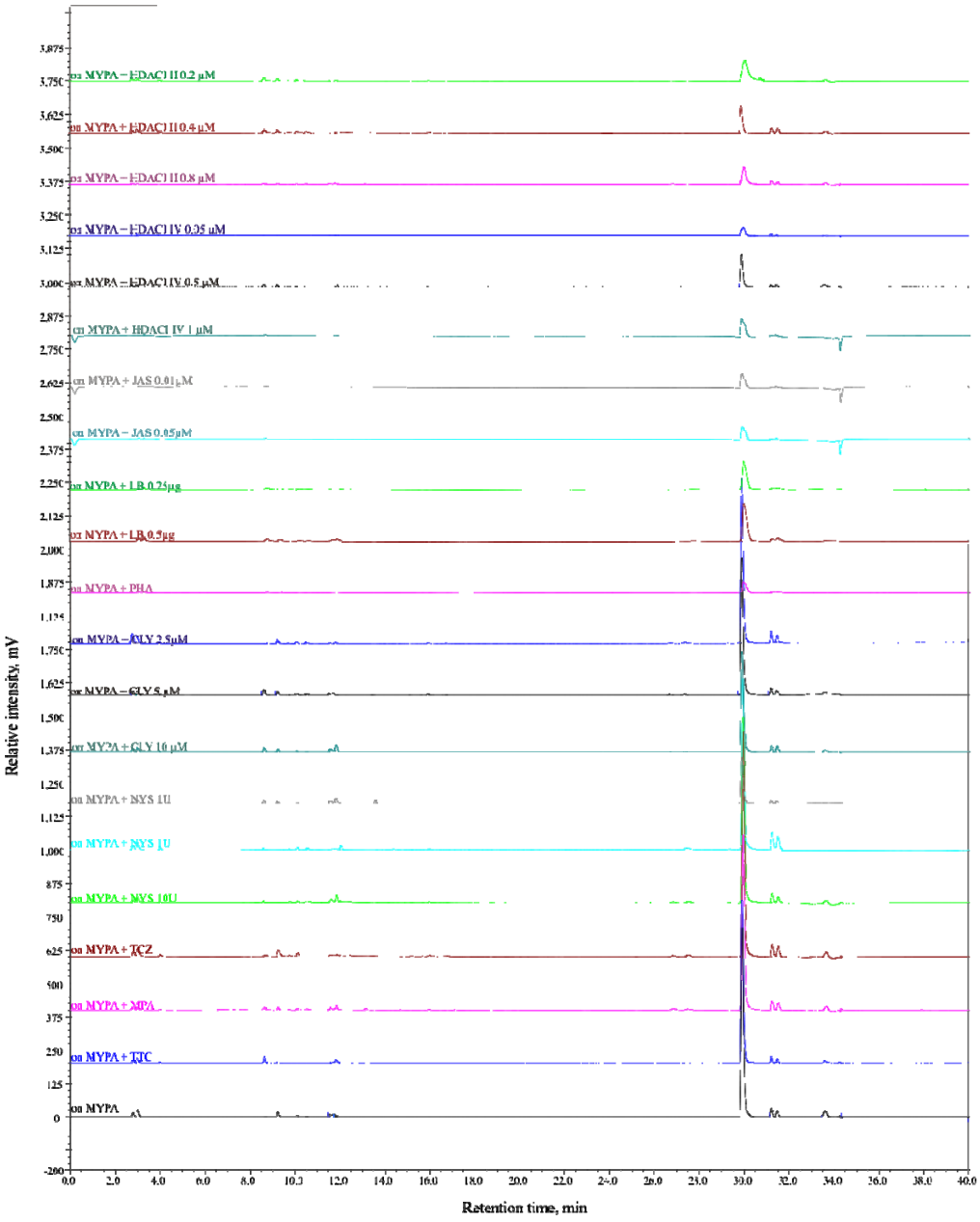


Figure 5.126: HPLC traces of extracts from *C. trigonosporum* cultured on MYPA at 20°C for 20 days with addition of different elicitors.

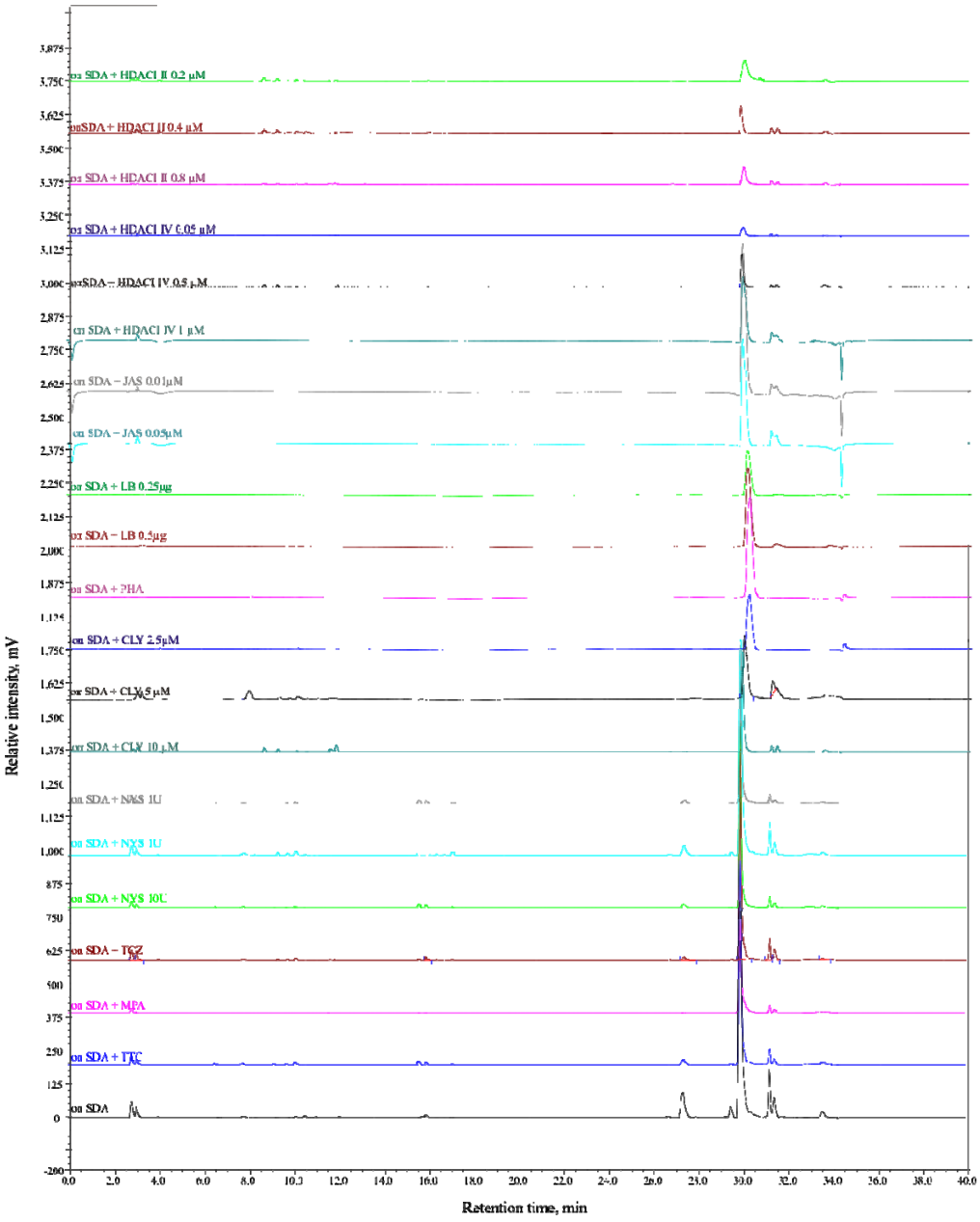


Figure 5.127: HPLC traces of extracts from *C. trigonosporum* cultured on SDA at 20°C for 20 days with addition of different elicitors.

Part F: *Penicillium* sp.

5.18 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 904_5@30.2 was also isolated from soil collected from Tiromoana Bush after treatment with 5% phenol. Morphological characteristics of the isolate 904_5@30.2 are shown in **Figure 5.128**. Colonies on MYPA medium attained 30-35 mm diameter in ten days at 20 °C and 60-65 mm diameter in 20 days. Mycelia were pale yellow to light brown, conidiogenesis abundant, greenish grey, reverse brown (**Figure 5.128a**) Culture on SDA led to a colony 20-25 mm diameter after ten days at 20 °C and 40-45 mm in 20 days, mycelium were white, conidiogenesis moderate, dark green, reverse pale brown (**Figure 5.128b**). Conidiophores were borne from aerial hyphae, stipes 20-150 x 2.0-2.5 µm, smooth walled, monoveriticillate. Phialides in verticils of four, ampulliform, 4-6 x 2.0-2.5 µm (**Figure 5.128c**); conidia subspheroidal to ellipsoidal, 1.5-2.0 µm diameter with smooth walls, borne in irregular chains (**Figure 5.128d**). A comparison of colony and morphological features with the description by Pitt (1979) enabled strain 904_5@30.2 to be identified as a *Penicillium* sp.

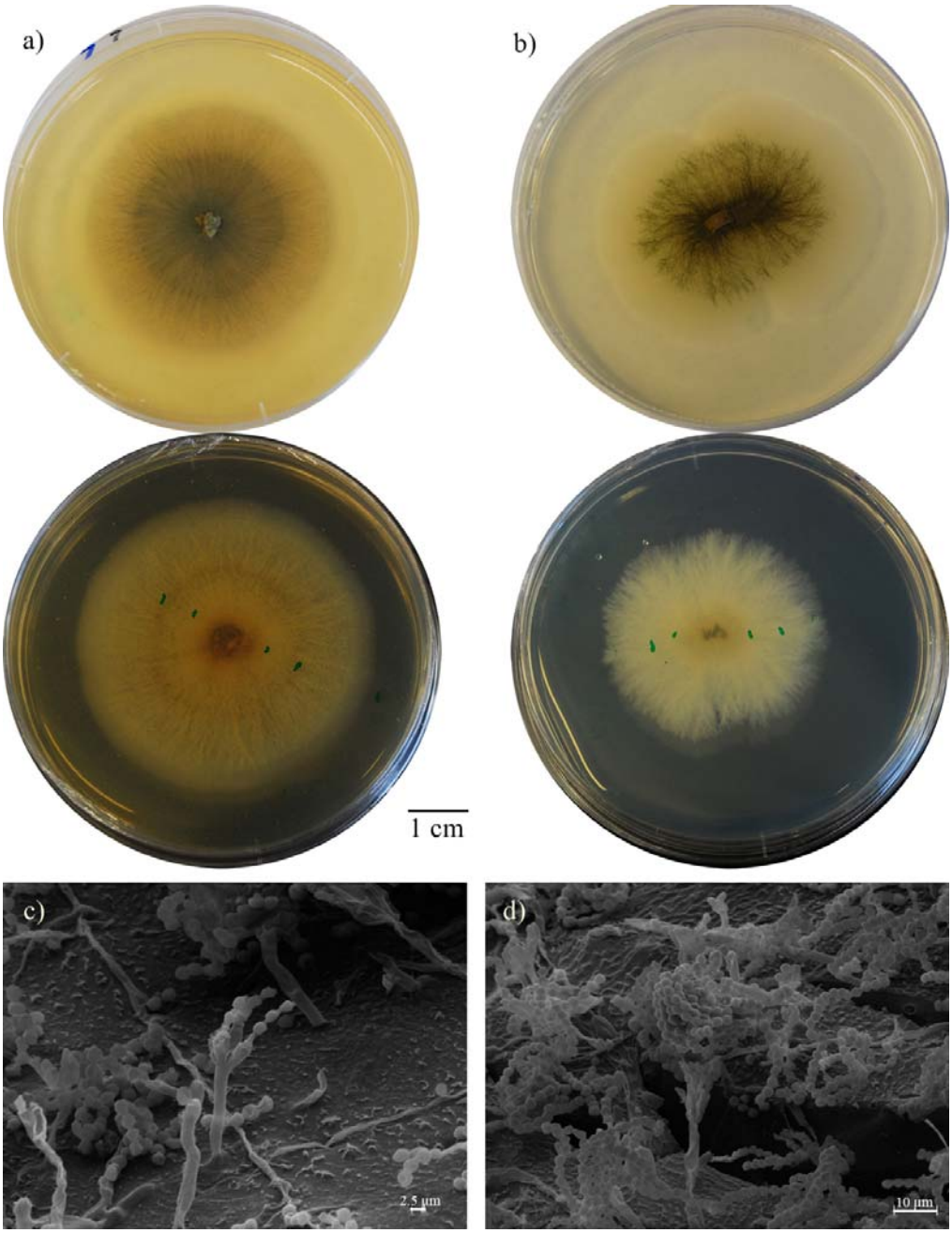


Figure 5.128: *Penicillium* sp. **a)** 20 day colony on MYPA plate at 20 °C; **b)** 20 day colony on SDA plate at 20 °C; **c)** SEM of conidiophores and conidia; **d)** conidia.

5.19 EFFECT OF THE ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.19.1 Effect of the elicitors on growth of *Penicillium* sp.

Penicillium sp. was grown on MYPA and SDA media at 20 °C for 20 days. Colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of mycelial growth of *Penicillium* sp. in the presence of elicitors in MYPA and SDA media is shown in **Figures 5.129** and **5.130** respectively.

It was noted that no growth was observed in the presence of cycloheximide and nystatin (100 U and 10 U concentrations) in the media. Growth of the fungus was greater on MYPA than SDA and that the growth rate declined after 15 days incubation. In the presence of elicitors, growth on MYPA was suppressed by LB (0.5 and 0.25 μg), while growth on SDA was suppressed by LB (0.5 and 0.25 μg) and MPA. A small enhancement of growth was found when TCZ and Jas (0.01 μM) were present in the media.

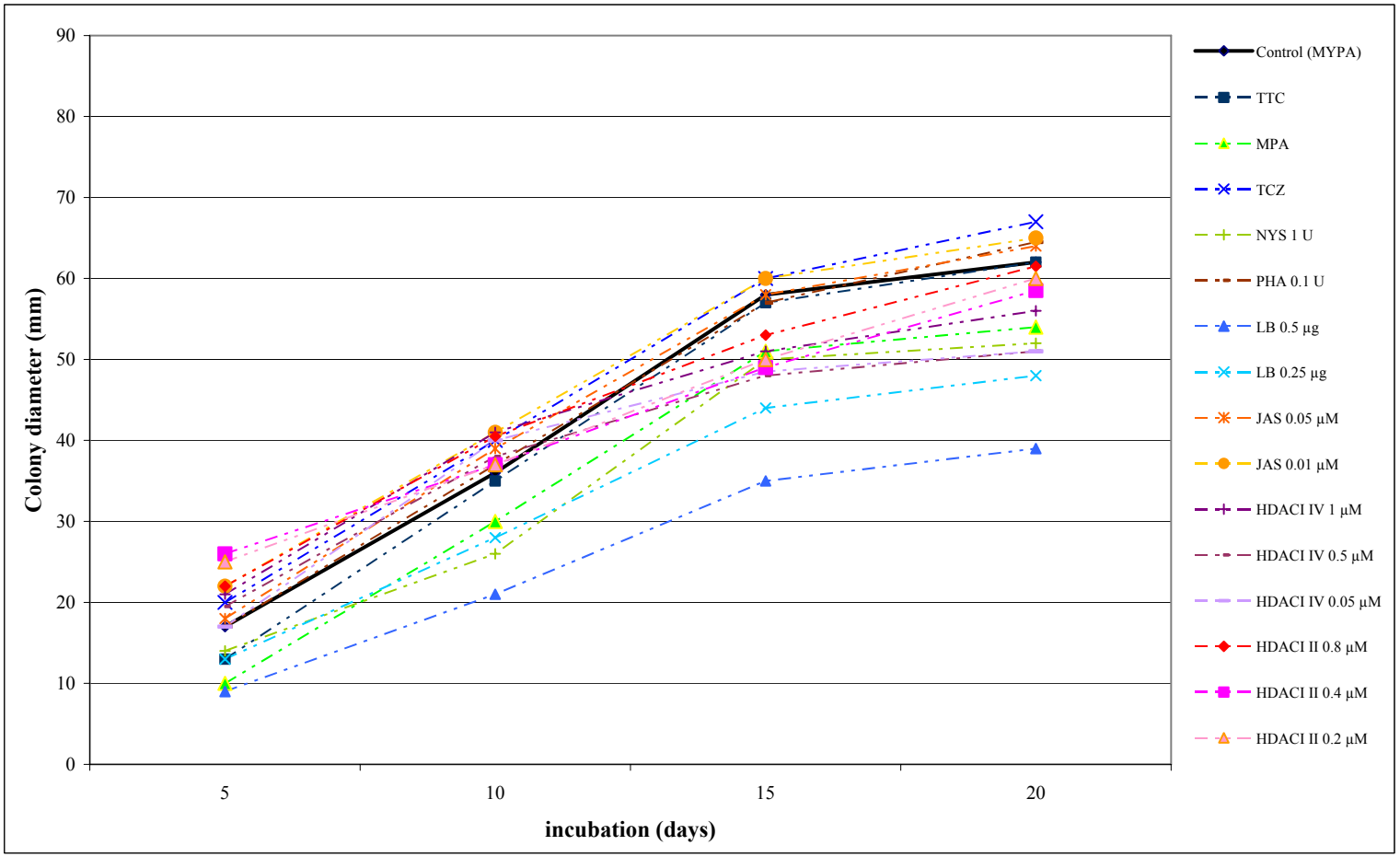


Figure 5.129: Effect of elicitors on the growth of *Penicillium* sp. on MYPA at 20 °C

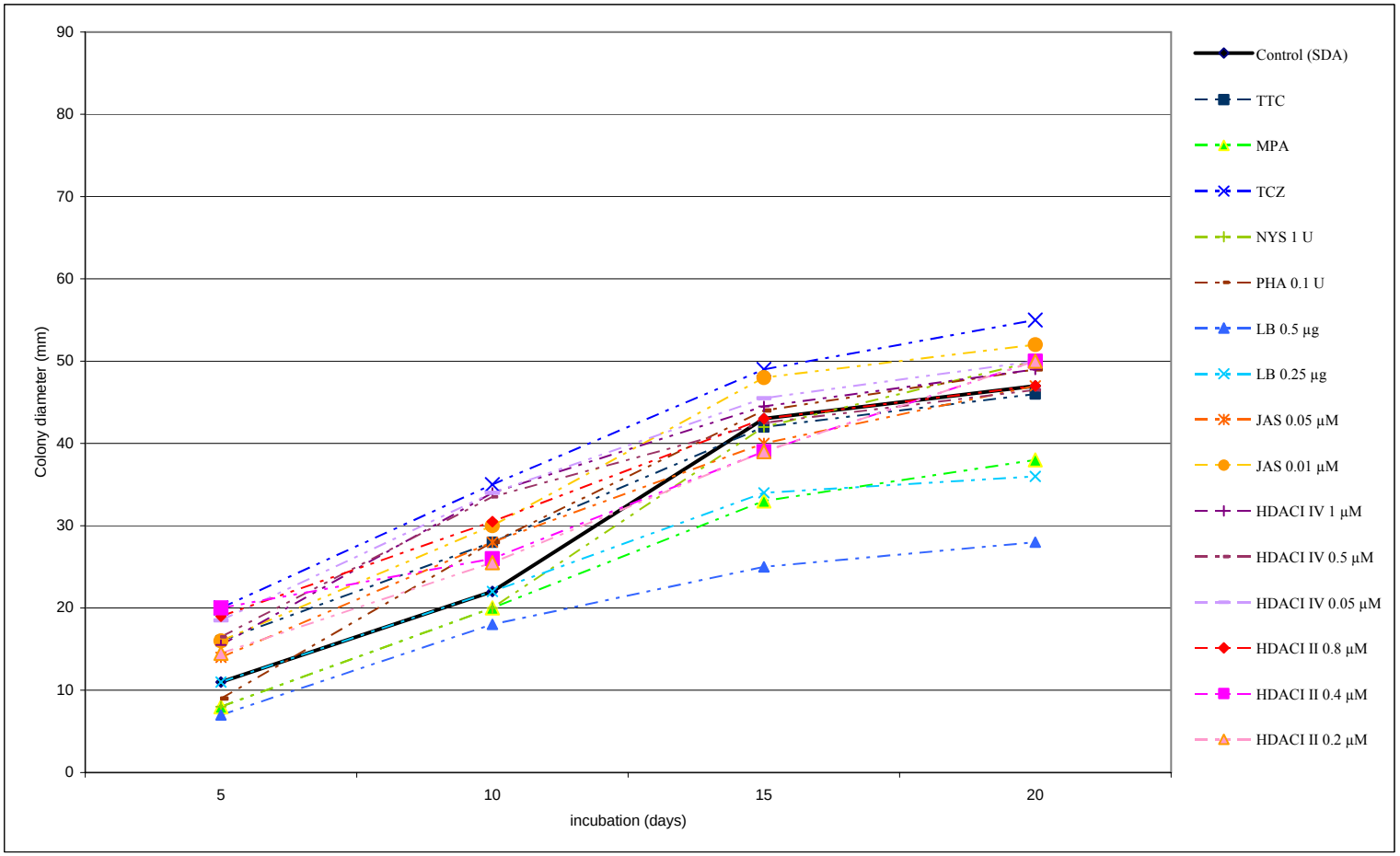


Figure 5.130: Effect of elicitors on the growth of *Penicillium* sp. on SDA at 20 °C

5.19.2 Effect of elicitors on cytotoxicity and metabolite production

5.19.2.1 Effect of the elicitors on the cytotoxicity of extracts

The P388 cytotoxicity of the extracts from each treatment is shown in **Table 5.22**. The extract from MYPA without any addition of elicitor was considered as being active (IC_{50} <12,500), while the rest showed no cytotoxicity.

Table 5.22: Effect of the elicitors on the cytotoxicity of the extracts from *Penicillium* sp on MYPA and SDA media (20 °C for 20 days).

Elicitors	Cytotoxicity on both media			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9270	9,802	F9285	>12,500
TTC	F9271	>12,500	F9286	>12,500
MPA	F9272	>12,500	F9287	>12,500
TCZ	F9273	>12,500	F9288	>12,500
NYS 1 U	F9276	>12,500	F9291	>12,500
PHA 0.1 U	F9280	>12,500	F9295	>12,500
LB 0.5 µg	F9281	>12,500	F9296	>12,500
LB 0.25 µg	F9282	>12,500	F9297	>12,500
JAS 0.05 µM	F9283	>12,500	F9298	>12,500
JAS 0.01 µM	F9284	>12,500	F9299	>12,500
HDACI IV 1 µM	F9449	>12,500	F9456	>12,500
HDACI IV 0.5 µM	F9451	>12,500	F9458	>12,500
HDACI IV 0.05 µM	F9453	>12,500	F9460	>12,500
HDACI II 0.8 µM	F9542	>12,500	F9548	>12,500
HDACI II 0.4 µM	F9544	>12,500	F9550	>12,500
HDACI II 0.2 µM	F9546	>12,500	F9552	>12,500

5.19.2.2 HPLC screening of the extracts

A comparison of ELSD traces showed no significant metabolite peaks present in extracts from *Penicillium* sp. when grown on MYPA and SDA with any elicitor (**Figures 5.131** and **5.132**)

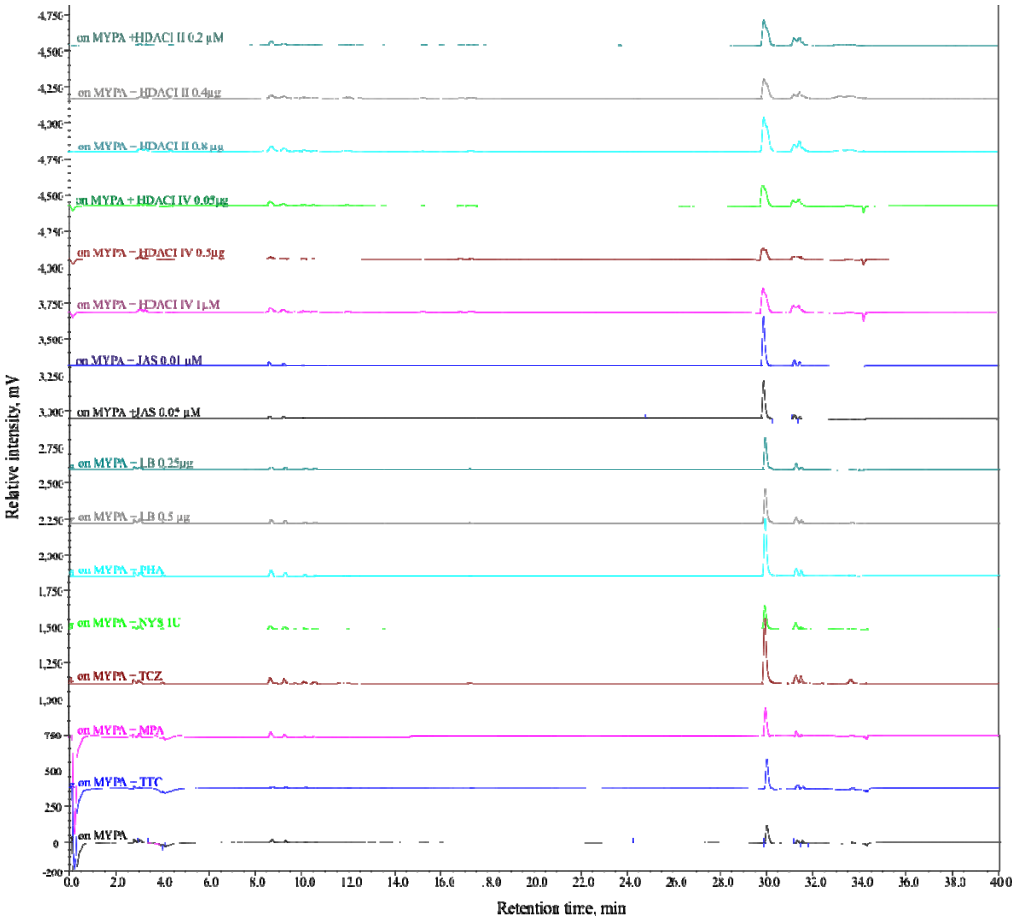


Figure 5.131: HPLC traces of extracts from *Penicillium* sp. cultured on MYPA at 20°C for 20 days with addition of different elicitors.

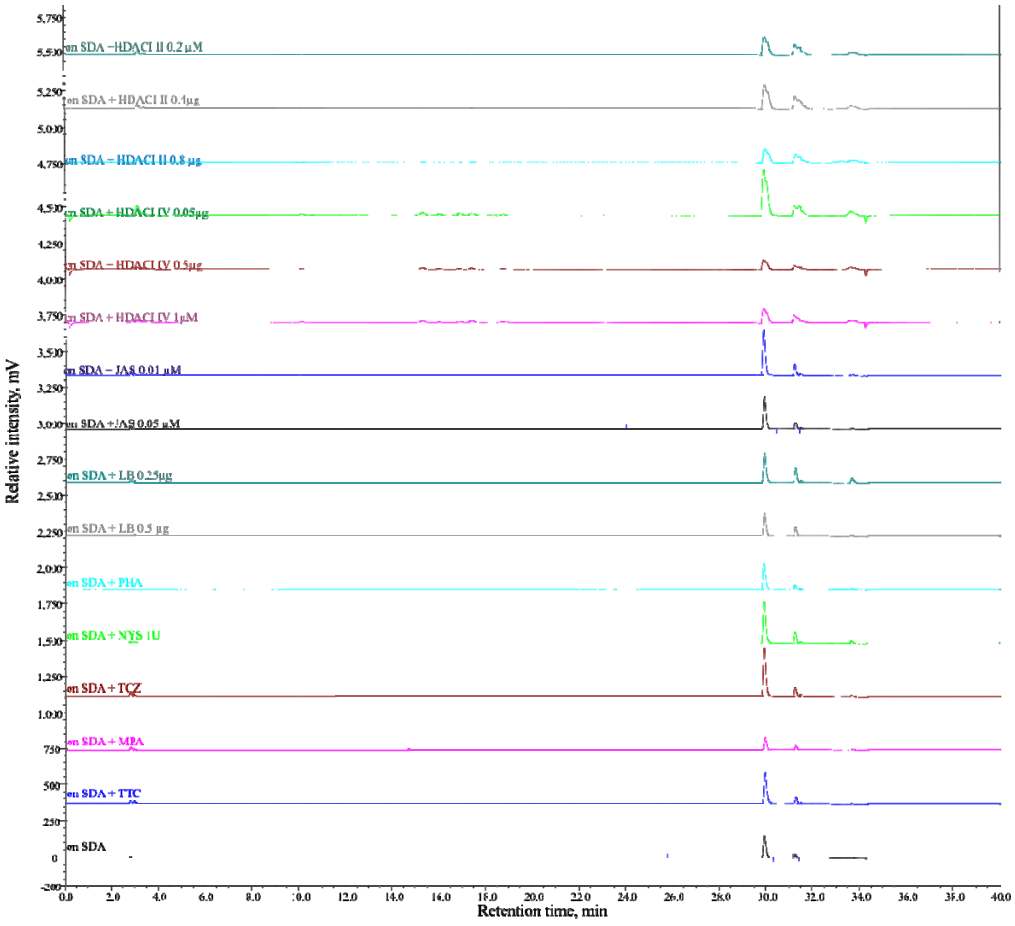


Figure 5.132: HPLC traces of extracts from *Penicillium* sp. cultured on SDA at 20°C for 20 days with addition of different elicitors.

Part G: Unidentified culture 901_7@20.14.

5.20 CULTURE CHARACTERISTICS AND MORPHOLOGY

Fungal strain 901_7@20.14 was isolated from soil collected from Tiromoana Bush after treatment with 7% phenol. Culture on SDA medium: 30-35 mm diameter in 10 days at 20 °C; mycelium white, reverse creamy (**Figure 5.133a**), culture reaching 70-75 mm diameter in 20 days (**Figure 5.133b**). The culture remained sterile during studies and hence a positive identification could not be made using cultural characteristics.

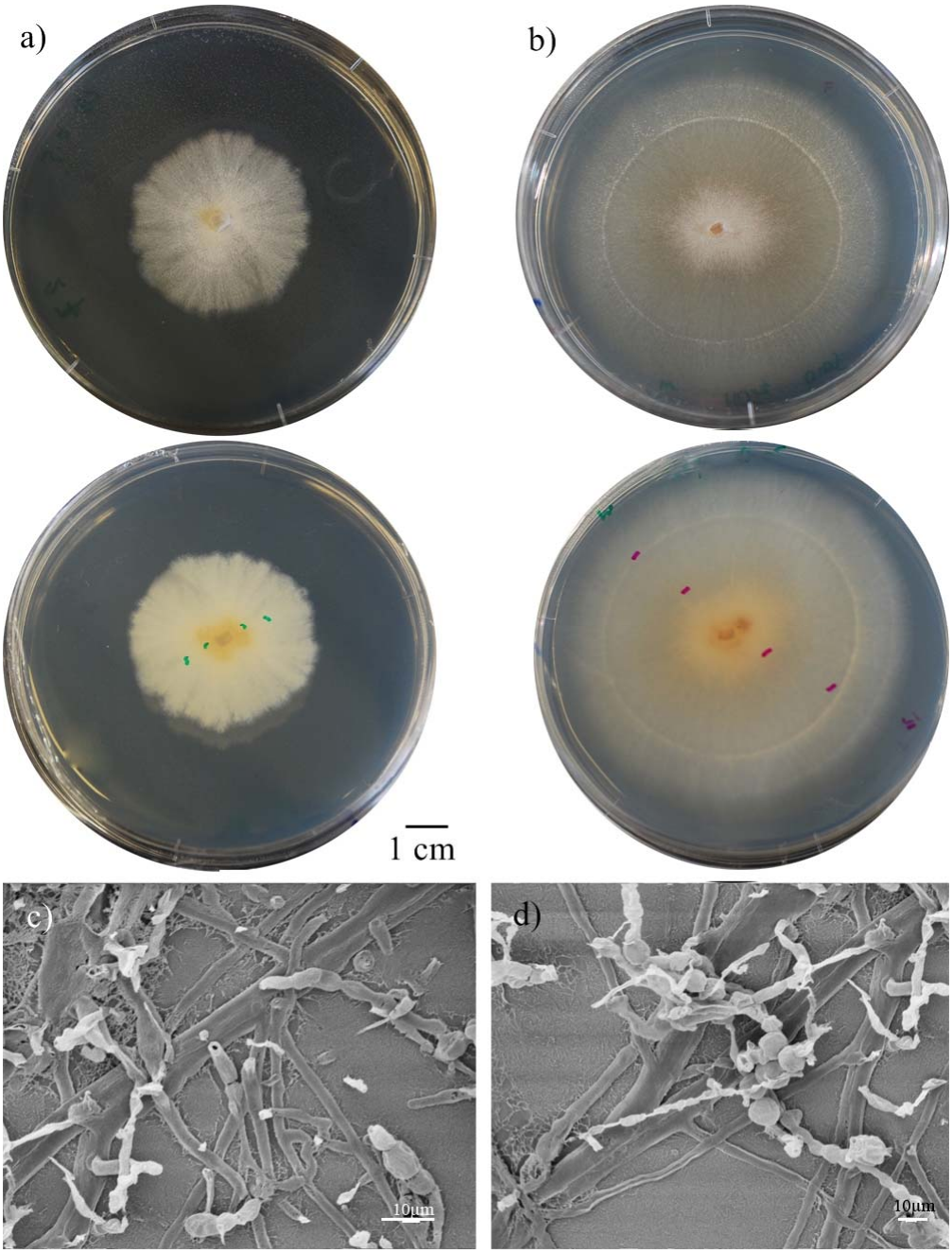


Figure 5.133: Isolate 901_7@20.14. **a)** 10 d colony on SDA at 20 °C; **b)** 20 day colony on SDA at 20 °C; **c-d)** SEM of hyphae.

5.21 EFFECT OF ELICITORS ON GROWTH AND METABOLITE PRODUCTION

5.21.1 Effect of elicitors on growth of 901_7@20.14

Fungal isolate 901_7@20.14 was grown on MYPA and SDA media at 20 °C for 20 days. Colony diameter (three replicates) was measured every 5 days for 20 days prior to extraction. A comparison of mycelial growth of the fungus in the presence of elicitors in MYPA and SDA is shown in **Figures 5.134** and **5.135** respectively.

No growth was observed in the presence of cycloheximide and nystatin (100 U and 10 U concentrations). No difference between the growth on MYPA and SDA was observed. Suppression of growth was found in the presence of LB (0.5 and 0.25 μg) and MPA in both media.

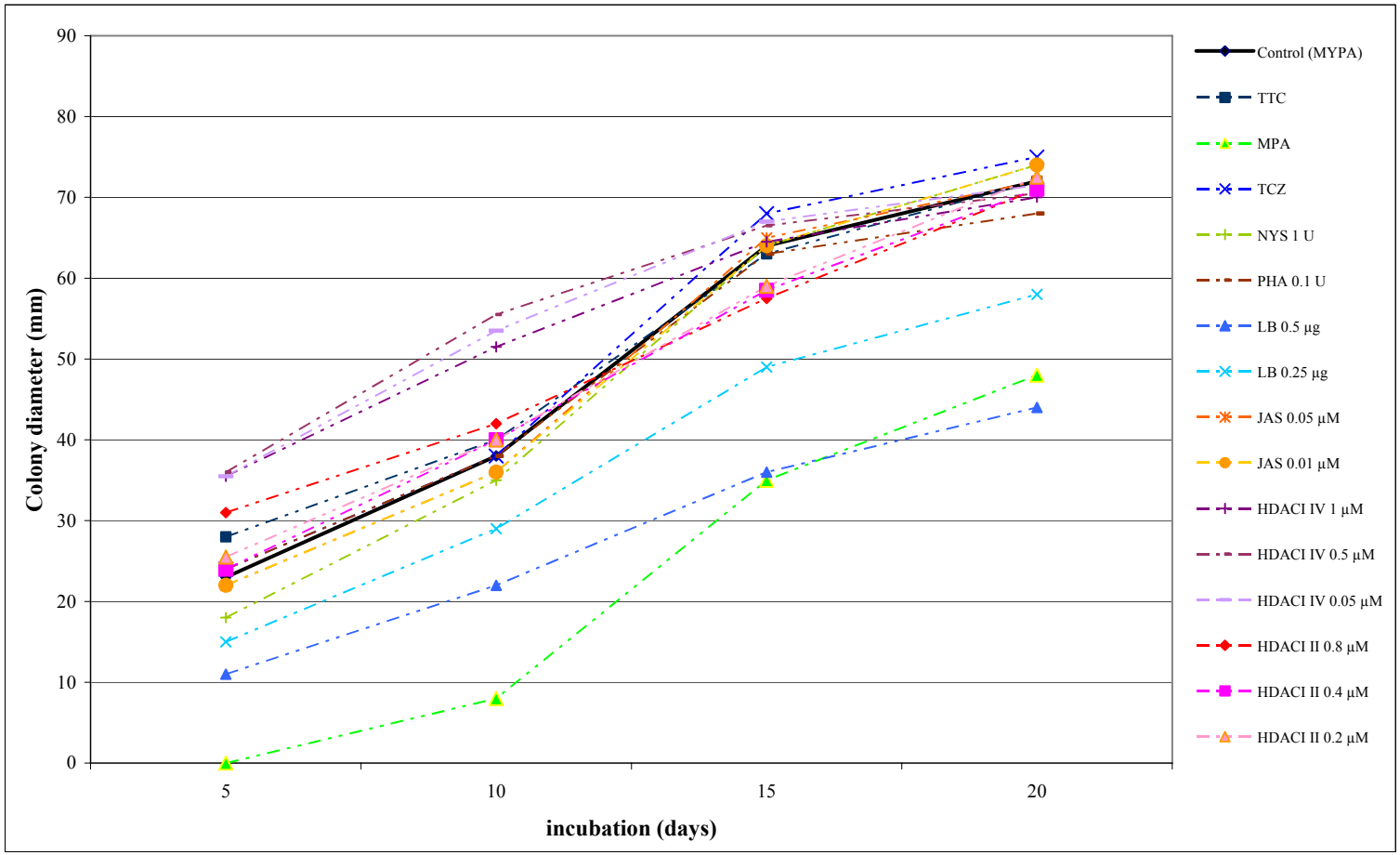


Figure 5.134: Effect of elicitors on growth of isolate 901_7@20.14 on MYPA at 20 °C

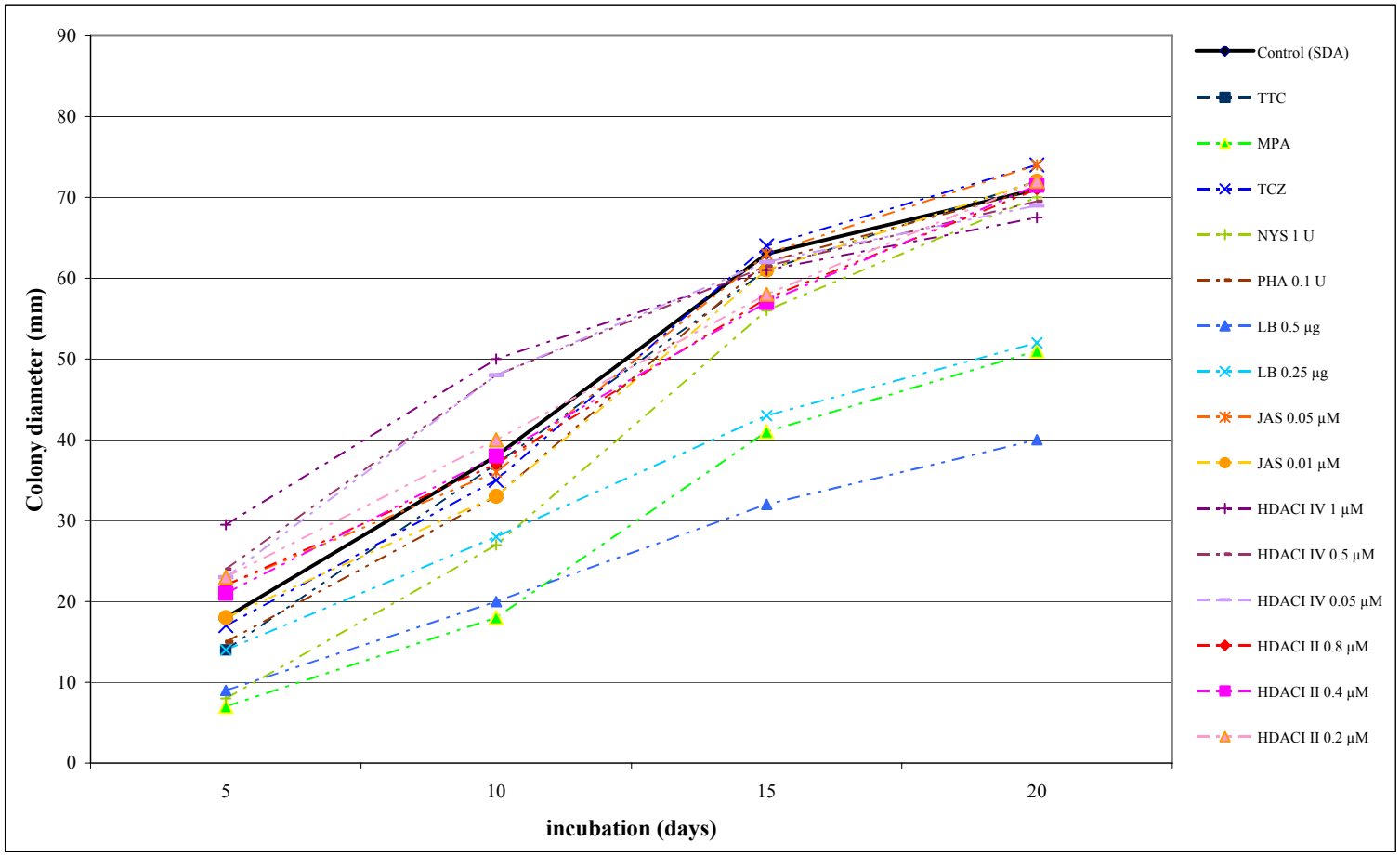


Figure 5.135: Effect of elicitors on growth of isolate 901_7@20.14 on SDA at 20 °C

5.21.2 Effect of the elicitors on cytotoxic and metabolite production

5.21.2.1 Effect of the elicitors on cytotoxicity of extracts

P388 cytotoxicity of extracts from each treatment is shown in **Table 5.23**. The extracts from MYPA and SDA without addition of elicitor were considered as being active against P388 cells (IC_{50} <12,500), while the remainder showed no cytotoxicity.

Table 5.23: Effect of elicitors on cytotoxicity of extracts from isolate 901_7@20.14 on MYPA and SDA media (20 °C for 20 days).

Elicitors	Cytotoxicity on both media			
	MYPA		SDA	
	Extracts	IC_{50} (ng/mL)	Extracts	IC_{50} (ng/mL)
Control	F9270	11,032	F9285	9,014
TTC	F9271	>12,500	F9286	>12,500
MPA	F9272	>12,500	F9287	>12,500
TCZ	F9273	>12,500	F9288	>12,500
NYS 1 U	F9276	>12,500	F9291	>12,500
PHA 0.1 U	F9280	>12,500	F9295	>12,500
LB 0.5 µg	F9281	>12,500	F9296	>12,500
LB 0.25 µg	F9282	>12,500	F9297	>12,500
JAS 0.05 µM	F9283	>12,500	F9298	>12,500
JAS 0.01 µM	F9284	>12,500	F9299	>12,500
HDACI IV 1 µM	F9449	>12,500	F9456	>12,500
HDACI IV 0.5 µM	F9451	>12,500	F9458	>12,500
HDACI IV 0.05 µM	F9453	>12,500	F9460	>12,500
HDACI II 0.8 µM	F9542	>12,500	F9548	>12,500
HDACI II 0.4 µM	F9544	>12,500	F9550	>12,500
HDACI II 0.2 µM	F9546	>12,500	F9552	>12,500

5.21.2.2 HPLC screening of the extracts

An examination of the ELSD HPLC traces showed that no significant metabolite peaks present in the extracts from 901_7@20.14 when grown on MYPA and SDA with any elicitor (**Figures 5.136** and **5.137**)

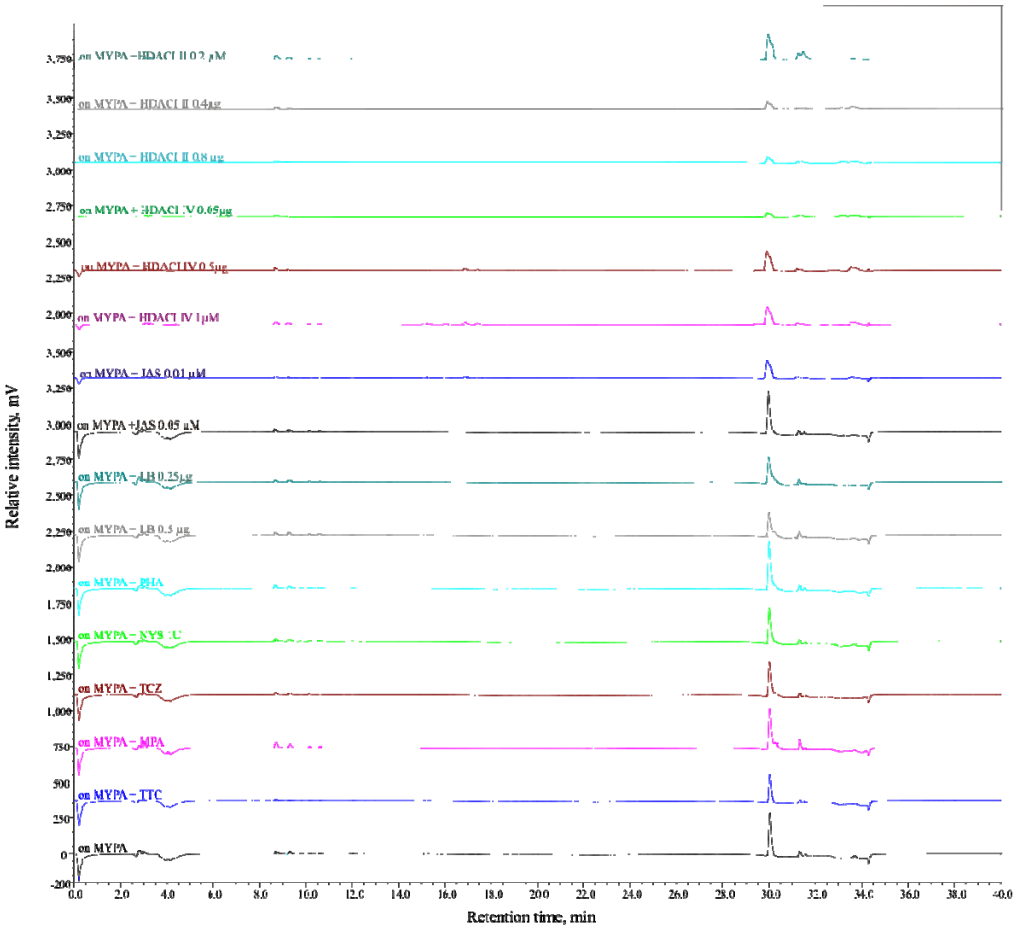


Figure 5.136: HPLC traces of extracts from isolate 901_7@20.14 cultured on MYPA at 20°C for 20 days with addition of different elicitors.

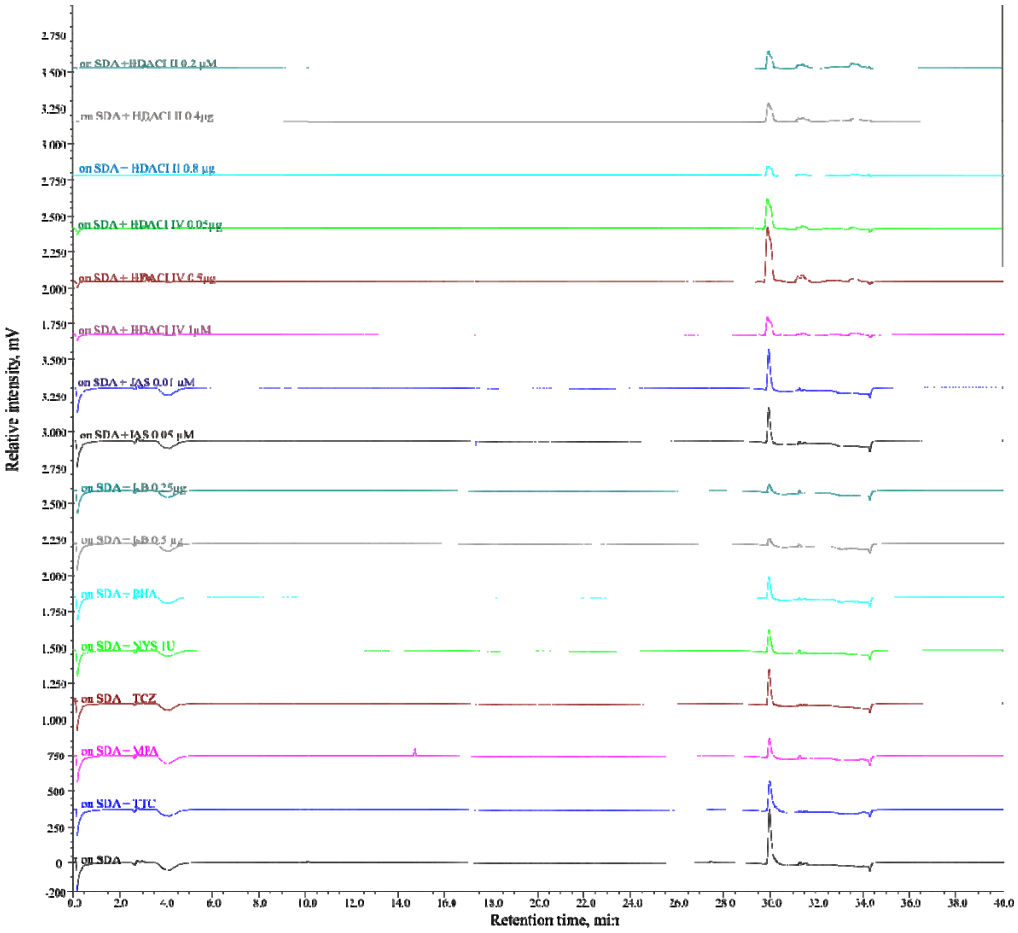


Figure 5.137: HPLC traces of extracts from isolate 901_7@20.14 cultured on SDA at 20°C for 20 days with addition of different elicitors.

5.22 DISCUSSION

In the study of the effect of culture conditions on metabolite production with *P. zonatum* and *C. globosum* it was established that the optimum temperature for cytotoxic metabolite production was 20 °C (see **Table 5.2**), which was lower than the optimum temperature for growth (25 °C) (see **Figures I** and **II** in **Appendix III**). This was consistent with the previous studies of Weinberg (1974) and Votruba and Vanek (1989) who found that the optimal temperature for secondary metabolites was often lower than that for growth of the producing microorganism. This phenomenon was explained by Farrell and Rose (1967) and Demian (1968) who commented that cultivation of microorganisms at sub-optimal temperature reduced the metabolic process of the microorganisms and hence the demand for the substrate supporting primary metabolism was also decreased. Therefore, this rapid growth rate will result in a low secondary metabolite yield.

The phase of fermentation was also another important factor for metabolite productions. Liquid fermentation is favoured by many researchers, especially for those who work in large-scale fermentation, or work with bacteria and actinomycete cultures. The fact that the control of fermentation in liquid culture is simpler than for solid culture and less labour is required probably accounts for this preference (Espinosa and Webb, 2003). In this study, however, the cytotoxicity of *P. zonatum, C. globosum* and *C. trigonosporum* was dominant when grown on solid media. These results conform with those reported by Ooijkaas *et al.* (2000), Robinson *et al*. (2001), Hölker *et al.* 2004

and Barrios *et al*. (2005) who found that fungal metabolite production was better on solid state than liquid state fermentation. Hölker *et al.* (2004) also pointed out that Ascomycetes and Basidiomycetes spent their evolutionary history as terrestrial organisms, therefore enzymes, secondary metabolites and spores were developed to be optimized to a moist solid substrate and not a liquid. Consequently, the cultivation of Ascomycetes in aqueous suspension may in fact impair their metabolic efficiency. Furthermore, Robinson *et al*. (2001) and Barrios *et al*. (2005) also stated that the oxygen transfer in liquid medium is very low due to the viscosity of the media and hence interferes with the yield of the metabolite production of the fungi.

During the study of the effect of elicitors on metabolite production a correlation between ascomata development and production of metabolites from the fungi was observed. Observations on ascomata production on *P. zonatum*. *C. globosum* and *Xylaria* sp. showed that in the presence of elicitors (such as HDACI II and HDACI IV) ascomata production was suppressed. These were the cultures that showed poor cytotoxicity and low metabolite production compared with cultures where ascomata production was not effected by elicitors. This phenomenon has been explained by Bennett and Cegler (1983) and Deacon (2006) as both the process of morphogenesis **and** the production of secondary metabolites starts at the end of exponential growth phase. Many other studies have also found that secondary metabolites in fungi are frequently associated with differentiation and sporulation and in most cases the secondary metabolites are not produced during log phase growth, but rather occur during the stationary phase (Adams and Yu, 1998,

Barrios *et al.* (2005) and Zain *et al*., 2009). For future studies in this area a definite consideration might be to allow a longer cultivation period for those cultures for which ascomata production had been suppressed by elicitors. This longer cultivation period might allow ascomata production to occur and with it possible metabolite production. Nevertheless, it would also be time consuming and would lead to unpredictable observations in those cases where the production of ascomata was permanently suppressed by elicitors.

It is believed that the production of secondary metabolites is affected by external stimuli such as environmental stress, competition and infection (Strohl, 2000). In this study, the production of poly-3-hydroxybutyrate (PHB) in *Bombardia* sp. was observed in the presence of elicitors in MYPA. PHB is produced by microorganisms, especially bacteria, for use as an intracellular carbon source, as an energy storage material and as storage of reductase equivalents (Rueping *et al.* 1998 and Wendlandt *et al.* 2001). In this case the production of PHB could have been a response to conditions of physiological stress (López *et al.* 1998).

Many studies in the past clearly showed that the presence of elicitors in the medium at subinhibitory concentration were found to enhance and modulate the production of secondary metabolites. For example, tetracycline was used to induce the production of phenazine in *Streptomyces* sp. (Mitova *et al.* 2008[a]), or the use of jasplakinolide to induce a production of chaetoglobosin from *Phomospis asparagi* (Christian *et al.* 2005). These studies were performed in liquid culture media. Nevertheless, the effect of these elicitors was also observed in the solid media in this study.

Four out of the seven selected fungi in this study clearly showed that metabolite production was effected by either media composition, elicitors or both in combination. The HPLC traces for *P. zonatum* (see **Figures 5.10** and **5.11**) showed that no modulation in metabolite productions had occurred, but an enhancement of metabolite production was observed in the presence of elicitors on both media. As for *C. globosum*, modulation and enhancement were observed in the presence of elicitors in both media (**Figures 5.38** and **5.39**). The media compositions were found to play a major role in the metabolite production for *Xylaria* sp. and *Bombardia* sp. Metabolite production was maximised when *Xylaria* sp. was grown on SDA rather than MYPA media (**Figures 5.69** and **5.70**), while in *Bombardia* sp. the metabolite production was maximised on MYPA, not SDA media (**Figures 5.93** and **5.94**). This could have been a result of the differences in the carbon and nitrogen sources in the media. In MYPA the main carbon source is the malt extract and the main nitrogen sources are peptone and the yeast extract, while in SDA the carbon source is dextrose and the main nitrogen source is the yeast extract (Atlast, 1993). This assumption was perhaps confirmed by the early study of Olutiola and Cole (1977) who found that differences in carbon and nitrogen sources could effect the growth and differentiation of fungi.

The careful selection of fungal strains should not be neglected for OSMAC studies. As stated earlier by Bode *et al.* (2000[b]) fungi or bacteria that produce secondary metabolites often have the potential to biosynthesise many alternative compounds from a single strain. This tendency was clearly demonstrated by *C. globosum* and *Xylaria* sp. in this study as they showed a

modulation in metabolite production in the presence of elicitors. These two fungal strains were recognized as the reproductive strain for biologically active secondary metabolite production. For example, metabolites such as azaphilones, chaetoglobosins and chaetoviridins are produced by *C. globosum* (Probst and Tamn, 1981; Takahashi *et al*. 1990; Jiao *et al*. 2004; Ding *et al*. 2005 and Park *et al.* 2005). For *Xylaria* sp. the bioactive metabolites would include the xyloketal derivatives, the antimalarial benzoquinones, the antifungal multipolides, and the cytotoxic metabolites such as the cytochalasins and tetralone derivatives (Boonphong *et al.* 2001; Wu *et al.* 2005; Tansuwan *et al*. 2007; Gu and Ding, 2008 and Pongcharoen *et al.* 2008). Surprisingly *Penicillium* sp., which was also recognized as a productive species (Ariyo *et al.* 1996) did not produce any metabolites at all in this study. This could have been due to strain selection up to the species level.

In most cases, the presence of LB (0.5 µg) or HDACI II and HDACI IV at all concentrations was found to suppress metabolite production. Despite the association between metabolite production and ascomata production mentioned above, another possibility could be that the concentrations of the elicitors used in this study were too high and beyond the subinhibitory point. For future studies the focus would be to obtain a single strain of a fungus and this strain would be treated with a wider variation of elicitor concentrations.

Chapter 6

Conclusions and future work

As part of the investigation into means of culturing less readily isolatable fungi from soil, especially Ascomycetes, a series of experiments were performed with the aim of increasing the opportunity to discover novel bioactive metabolites. These experiments were guided by a set of aims.

The first aim was to develop a selective isolation technique for Ascomycetes. Once developed the technique would be used on soil samples selected from around New Zealand. A phenol pasteurization technique was successfully developed and a total of 407 isolates were obtained from 2-8% phenol treatments. Analysis of results indicated that treatment with 3% and 4% phenol were the most useful concentration for partial pasteurization of the soil. At these concentrations a greater variety of fungal isolates and Ascomycetes were more frequently isolated. A 2% phenol elimination of the fast growing mucoraceous fungi was not successful. As for higher phenol treatments (5% to 8%), fewer fungal isolates were retrieved and of those that were, most had also been retrieved at lower phenol levels.

The second aim was to discover which of the isolates was producing bioactive compounds. This could be either cytotoxicity, antifungal or antimicrobial activity. Of the 407 isolates, 123 were active against P388

cells and 40 extracts were active against *B. subtilis* (<20% cell viability) and 19 active against *C. albicans* (<20% cell viability). None of the extracts were active against *P. aeruginosa*. Of the total of 123 extracts that were active against P388 cell in the quick screen assay, 87 extracts showed IC_{50} values <12,500 ng/mL. These were selected for further investigation.

Thirdly, a chemical characterization of metabolites in active extracts was undertaken. The structural elucidation of the metabolites of interest was greatly aided by the in-house dereplication techniques consisting of a combination of HPLC-UV/R_t database, MS analysis, the CapNMR technique and the AntiMarin Database. A significant advantage was gained by the use of the CapNMR technique which enabled full NMR characterization on very small quantities of pure compounds (<20 µg). From the dereplication studies, 48 known compounds were identified from the active extracts. For a summary of the compounds identified from the active extracts see **Table 4.2**. Two new metabolites, F7301-1 (**Figure 5.28**) and F7301-2 (**Figure 5.31**) were isolated and identified from *Pseudeurotium zonatum.* This was a fungal isolate that had been retrieved from a soil sample treated with 4% phenol.

Finally, manipulation of the metabolite profile of seven selected fungi was undertaken. This work was based on the OSMAC (one strain many compounds) and hormetic (low-dose stimulation and high-dose inhibition) concept. Of these seven chosen fungi, three isolates (*Chaetomium globosum*. *Xylaria* sp. and *Bombardia* sp.) could have their secondary metabolite production altered by use of subinhibitory concentrations of elicitors. In the

case of *C. globosum*, tetrahydrofuran A and tetrahydrofuran B were the main compounds produced when the fungus was grown on media without elicitors. Production of chaetoviridin A and chaetoglobosin A were induced when this isolate was treated with certain elicitors (summary in **Table 5.2**).

Xylaria sp. was found to produce more metabolites when it was grown on SDA than MYPA indicating the importance of the growth medium for metabolite studies in addition to elicitors. The known compound ternatin was the main compound isolated from this strain on SDA without elicitors. With elicitors (as listed in **Table 5.16)**, an enhancement in the production of 2-hexylidene-3-methylsuccinic acid, dechlorogriseofulvin and griseofulvin was observed and production of chaetoglobosin E also induced. Compounds cyclo[L-*trans*-4-hydroxyprolinyl-L-leucine] and cyclo[D-*cis*-4-hydroxylprolyl-D-phenylalanyl] were isolated from *Bombardia* sp. when it was grown on MYPA without elicitors. Eight metabolite peaks, which were subsequently identified to be a series of poly-3-hydroxybutyrates (n=3-10), were induced by all elicitors except LB. No metabolites were observed on SDA.

Enhancement of metabolite productions in *P. zonatum* was observed in the presence of certain elicitors, but there was no induction of new metabolite production. Finally, neither enhancement nor inducement were observed in *C. trigonosporum*, *Penicillium* sp. and isolate 901_7@20.14 when they were treated with elicitors.

The original aims, as outlined in the Introduction, were accomplished. It was perhaps disappointing that a greater range of new bioactive compounds were

not discovered and that it was difficult to accomplish the induction of new metabolites in the OSMAC experiments. The work conducted in this thesis was confined to a study of Ascomycetes species. The study has made a significant contribution in understanding how to better produce and manipulate secondary metabolites of the Ascomycetes species. The successful OSMAC studies that have been reported have mainly focused on the Actinomycetes not Ascomycetes. It is possible that Ascomycetes are not as easily manipulated under OSMAC conditions as are the Actinomycetes.

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Appendix I

Media for Isolation and Cultivation

MEDIA FOR ISOLATION METHOD

Chlortetracycline/streptomycin antibacterial solution

2.5 g streptomycin sulfate (Sigma) and 0.5 g chlortetracycline hydrochloride (sigma) were dissolved in 100 mL distilled water. The solution was sterile filtered and stored frozen at -20 °C.

¼ strength Sabouraud dextrose agar-chlortetracycline/streptomycin medium (¼SDA^{+})

3.9 g Sabouraud dextrose agar (Oxoid), 0.6 g yeast extract (Oxoid), and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water. The medium pH of 5.7 (±0.1) was measured and recorded prior to sterilization. Addition of 6.0 mL antibacterial solution was made to the media prior to plate preparation.

Appendix I

MEDIA FOR CULTIVATION METHOD

Malt yeast peptone agar (MYPA)

12.0 g malt extract (Oxoid), 1.8 g peptone (Oxoid), 0.6 g yeast extract (Oxoid) and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water. The medium pH was measured and recorded as pH 6.1 (±0.1) prior to sterilization

¼ strength Sabouraud dextrose agar (¼ SDA)

3.9 g Sabouraud dextrose agar (Oxoid), 0.6 g yeast extract (Oxoid) and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water. The medium pH was measured and recorded as pH 5.7 (±0.1) prior to sterilization.

MEDIA FOR ONE STRAIN MANY COMPOUNDS (OSMAC) APPROACH

Base media used in this experiment were MYPA and ¼ SDA. The elicitors, as outlined below, were added to the base media.

Tetracycline

A 25 mg/mL tetracycline (Sigma) stock solution was prepared in sterile distilled water. 24 µL was then added to 600 mL of base media to give a final concentration of 1 µg/mL of tetracycline in each agar plate.

Appendix I

Mycophenolic acid

A 25 mg/mL mycophenolic acid (Sigma) stock solution was prepared in methanol. 36 µL of stock solution was then added to 600 mL of based media to give a final concentration of 1.5 µg/mL of mycophenolic acid in each agar plate.

Tricyclazole

A 5 mg/mL tricyclazole (Sigma) stock solution was prepared in methanol. 180 µL of stock solution was then added to 600 mL of base media to give a final concentration of 1.5 µg/mL of tricyclazole in each agar plate.

Nystatin

A nystatin stock solution of 10000 U/mL (Sigma) was diluted to give a final concentration of 100 , 10 and 1U/mL of nystatin in each agar plate.

Cycloheximide

A 0.5 mM cycloheximide (Sigma) stock solution was prepared in sterile distilled water and diluted to give final concentrations of 10, 5 and 2.5 µM of cycloheximide in each agar plate.

Phalloidin

A stock phalloidin solution of 200 U/mL (Sigma) was diluted to give a final concentration of 0.1 U/mL of phalloidin in each agar plate.

Latrunculin B

A 1 mg/mL latrunculin B (Sigma) stock solution was prepared in methanol and diluted to give final concentrations of 0.5 and 0.25 µg/mL of latrunculin B in each agar plate.

Jasplakinolide

A 1 mM jasplakinolide (CALBIOCHEM) stock solution was prepared in methanol and diluted to give final concentrations of 0.05 and 0.01 µM of jasplakinolide in each agar plate.

Histone Deacetylase Inhibitor II

A 22 mM Histone Deacetylase Inhibitor II (CALBIOCHEM) stock solution was prepared in methanol and diluted to give final concentrations of 0.8, 0.4 and 0.2 µM of Histone Deacetylase Inhibitor II in each agar plate.

Histone Deacetylase Inhibitor IV

A 500 µM Histone Deacetylase Inhibitor IV (CALBIOCHEM) stock solution was prepared in methanol and diluted to give final concentrations of 1, 0.5 and 0.05 µM of Histone Deacetylase Inhibitor IV in each agar plate.

Appendix II

Colony count from each soil sample

Table I: Colony count from soil site 151.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	0	9	250	250	250	5%	5	0	0	0	0	0
	10	0	500	500	500	500		10	0	0	0	0	0
	15	500	500	500	500	500		15	0	0	0	0	0
	20	500	500	500	500	500		20	0	0	0	0	0
	25	500	500	500	500	500		25	0	0	0	0	0
	30	300	500	500	500	500		30	0	0	0	0	0
	37	0	300	500	500	500		37	0	0	0	0	0
2%	5	0	0	0	2	6	6%	5	0	0	0	0	0
	10	0	4	4	4	4		10	0	0	0	0	0
	15	1	5	55	55	55		15	0	0	0	0	0
	20	2	9	10	10	10		20	0	0	0	0	0
	25	4	5	5	5	5		25	0	0	0	0	0
	30	1	7	7	7	7		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
3%	5	0	0	0	1	1	7%	5	0	0	0	0	0
	10	0	1	1	1	5		10	0	0	0	0	0
	15	0	5	5	5	5		15	0	0	0	0	0
	20	1	10	11	11	11		20	0	0	0	0	0
	25	2	4	4	4	4		25	0	0	0	0	0
	30	0	1	1	1	1		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	1	3	8%	5	0	0	0	0	0
	10	0	0	0	1	2		10	0	0	0	0	0
	15	0	0	0	3	3		15	0	0	0	0	0
	20	0	1	1	5	5		20	0	0	0	0	0
	25	0	3	3	3	4		25	0	0	0	0	0
	30	0	2	2	4	4		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table II: Colony count from soil site 381.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	0	9	250	250	250	5%	5	0	0	0	0	0
	10	0	500	500	500	500		10	0	0	0	0	0
	15	500	500	500	500	500		15	0	0	0	0	11
	20	500	500	500	500	500		20	0	0	5	10	11
	25	500	500	500	500	500		25	0	0	0	0	0
	30	300	500	500	500	500		30	0	0	0	0	0
	37	0	300	500	500	500		37	0	0	0	0	0
2%	5	0	0	2	2	2	6%	5	0	0	0	0	0
	10	0	1	3	3	3		10	0	0	0	0	0
	15	0	3	3	5	5		15	0	0	0	0	18
	20	4	10	17	19	19		20	0	0	0	0	1
	25	2	5	8	9	9		25	0	0	0	0	0
	30	0	2	3	5	5		30	0	0	0	0	0
	37	2	2	2	2	2		37	0	0	0	0	0
3%	5	0	0	1	1	1	7%	5	0	0	0	0	0
	10	0	0	1	1	1		10	0	0	0	0	0
	15	0	0	1	3	3		15	0	0	0	0	6
	20	1	7	9	11	11		20	0	0	0	0	0
	25	0	1	5	8	8		25	0	0	0	0	0
	30	0	0	1	1	1		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	0	0	0		10	0	0	0	0	0
	15	0	2	2	2	2		15	0	0	0	0	0
	20	0	2	6	8	8		20	0	0	0	0	0
	25	0	1	4	8	8		25	0	0	0	0	0
	30	0	0	0	0	0		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table III: Colony count from soil site 418.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	0	0	101	101	101	5%	5	0	0	0	0	0
	10	0	36	243	243	245		10	0	0	0	0	0
	15	67	107	284	300	500		15	0	0	0	0	0
	20	100	203	300	500	500		20	0	0	0	0	0
	25	83	100	500	500	500		25	0	0	0	0	0
	30	52	70	84	100	100		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
2%	5	0	0	0	0	0	6%	5	0	0	0	0	0
	10	0	0	0	0	0		10	0	0	0	0	0
	15	0	0	0	0	0		15	0	0	0	0	0
	20	0	0	0	0	0		20	0	0	0	0	0
	25	0	0	0	0	0		25	0	0	0	0	0
	30	0	0	0	0	0		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
3%	5	0	0	0	0	0	7%	5	0	0	0	0	0
	10	0	0	0	0	0		10	0	0	0	0	0
	15	0	0	0	0	0		15	0	0	0	0	0
	20	0	0	0	0	0		20	0	0	0	0	0
	25	0	0	0	0	0		25	0	0	0	0	0
	30	0	0	0	0	0		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	0	0	0		10	0	0	0	0	0
	15	0	0	0	0	0		15	0	0	0	0	0
	20	0	0	0	0	0		20	0	0	0	0	0
	25	0	0	0	0	0		25	0	0	0	0	0
	30	0	0	0	0	0		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table IV: Colony count from soil site 456.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	500	500	500	500	500	5%	5	0	0	0	0	0
	10	500	500	500	500	500		10	0	0	0	0	0
	15	500	500	500	500	500		15	0	0	0	1	1
	20	500	500	500	500	500		20	0	0	0	0	0
	25	500	500	500	500	500		25	0	3	4	6	6
	30	500	500	500	500	500		30	0	0	0	0	0
	37	500	500	500	500	500		37	0	0	0	0	0
2%	5	0	0	0	8	8	6%	5	0	0	0	0	0
	10	0	9	9	15	15		10	0	0	0	0	0
	15	4	21	21	24	24		15	0	2	2	5	5
	20	2	29	32	32	32		20	0	4	4	5	5
	25	2	32	500	500	500		25	0	2	5	5	5
	30	1	5	500	500	500		30	0	2	3	3	3
	37	0	0	0	0	0		37	0	0	0	0	0
3%	5	0	0	0	0	0	7%	5	0	0	0	0	0
	10	0	0	0	26	26		10	0	0	0	1	1
	15	0	72	72	72	72		15	0	2	3	5	5
	20	0	46	63	63	63		20	0	0	0	0	0
	25	0	7	13	16	16		25	0	0	3	3	3
	30	0	2	2	2	2		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	0	7	7		10	0	0	0	0	0
	15	0	13	13	13	13		15	0	0	0	0	0
	20	0	10	12	12	12		20	0	0	0	0	0
	25	0	12	12	12	12		25	0	0	0	0	0
	30	0	4	6	6	6		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table V: Colony count from soil site 654.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	250	500	500	500	500	5%	5	0	0	0	0	0
	10	500	500	500	500	500		10	0	0	0	4	5
	15	500	500	500	500	500		15	0	0	0	0	0
	20	500	500	500	500	500		20	0	0	0	1	2
	25	500	500	500	500	500		25	0	0	0	1	1
	30	74	150	500	500	500		30	0	0	0	0	0
	37	18	500	500	500	500		37	0	0	0	0	0
2%	5	0	1	2	2	2	6%	5	0	0	0	0	0
	10	2	6	8	10	13		10	0	0	0	0	0
	15	4	10	12	13	17		15	0	0	0	0	1
	20	17	500	500	500	500		20	0	0	0	0	0
	25	3	500	500	500	500		25	0	0	0	0	0
	30	7	13	13	13	13		30	0	0	0	0	0
	37	1	2	2	2	2		37	0	0	0	0	0
3%	5	0	0	0	0	0	7%	5	0	0	0	0	0
	10	0	0	2	5	7		10	0	0	0	0	0
	15	1	2	4	4	5		15	0	0	0	0	0
	20	1	5	6	6	6		20	0	0	0	0	0
	25	0	5	5	5	5		25	0	0	0	0	0
	30	0	0	0	0	1		30	0	0	0	0	0
	37	1	1	1	1	1		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	3	6	6		10	0	0	0	0	0
	15	2	2	4	3	3		15	0	0	0	0	2
	20	0	1	1	1	3		20	0	0	0	0	0
	25	0	2	6	6	7		25	0	0	0	0	0
	30	0	3	3	4	4		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table VI: Colony count from soil site 701.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	0	500	500	500	500	5%	5	0	0	0	0	102
	10	500	500	500	500	500		10	0	0	0	0	113
	15	500	500	500	500	500		15	0	0	0	0	101
	20	500	500	500	500	500		20	0	67	107	110	110
	25	500	500	500	500	500		25	77	90	92	95	100
	30	65	300	500	500	500		30	67	105	107	110	113
	37	35	200	300	500	500		37	0	0	0	0	2
2%	5	0	0	37	76	76	6%	5	0	0	0	0	32
	10	0	0	40	46	49		10	0	0	0	0	93
	15	14	19	41	41	43		15	0	0	0	0	77
	20	6	12	12	14	14		20	0	60	92	93	93
	25	19	20	31	32	32		25	62	87	91	92	93
	30	0	6	18	20	20		30	39	95	97	100	107
	37	1	1	7	8	8		37	0	0	0	0	1
3%	5	0	0	20	57	60	7%	5	0	0	0	0	0
	10	0	0	30	73	74		10	0	0	0	0	3
	15	8	26	35	49	51		15	0	0	0	0	30
	20	28	38	43	56	57		20	0	35	73	73	73
	25	7	21	22	31	31		25	7	60	62	67	67
	30	0	26	27	31	31		30	28	69	75	79	64
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	40	50	50	8%	5	0	0	0	0	0
	10	0	0	40	64	65		10	0	0	0	0	0
	15	0	21	47	70	70		15	0	0	0	0	0
	20	20	40	43	60	60		20	0	0	0	10	11
	25	7	30	60	76	76		25	0	31	40	49	49
	30	0	31	31	47	47		30	0	38	40	42	47
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table VII: Colony count from soil site 702.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	5	6	500	500	500	5%	5	0	0	0	0	0
	10	200	500	500	500	500		10	0	0	0	0	68
	15	300	500	500	500	500		15	0	0	0	0	84
	20	300	500	500	500	500		20	0	0	12	52	67
	25	400	500	500	500	500		25	0	0	10	22	29
	30	50	500	500	500	500		30	0	0	5	7	7
	37	3	250	500	500	500		37	0	0	0	0	0
2%	5	0	0	0	0	8	6%	5	0	0	0	0	0
	10	0	0	0	3	7		10	0	0	0	0	35
	15	6	7	18	45	45		15	0	0	0	0	3
	20	15	47	50	50	58		20	0	0	9	42	59
	25	12	28	30	32	35		25	0	0	7	9	20
	30	50	5	10	11	11		30	0	0	0	0	0
	37	1	1	1	6	9		37	0	0	0	0	0
3%	5	0	0	0	0	9	7%	5	0	0	0	0	0
	10	0	0	0	54	62		10	0	0	0	0	0
	15	0	8	32	52	70		15	0	0	0	0	66
	20	2	37	42	55	71		20	0	0	7	40	49
	25	5	15	26	29	29		25	0	0	0	1	0
	30	4	4	14	19	14		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	0	27	8%	5	0	0	0	0	0
	10	0	0	0	53	70		10	0	0	0	0	0
	15	0	7	40	54	90		15	0	0	0	0	17
	20	2	29	30	32	55		20	0	0	0	1	1
	25	4	28	32	46	57		25	0	0	0	0	0
	30	3	15	20	20	20		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table VIII: Colony count from soil site 770.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	69	500	500	500	500	5%	5	0	0	0	0	0
	10	132	500	500	500	500		10	0	0	0	0	68
	15	300	500	500	500	500		15	0	0	0	0	84
	20	300	500	500	500	500		20	0	6	12	52	67
	25	300	500	500	500	500		25	0	4	10	22	29
	30	300	500	500	500	500		30	0	5	5	7	7
	37	300	500	500	500	500		37	0	0	0	0	0
2%	5	0	0	0	1	1	6%	5	0	0	0	0	0
	10	0	0	1	2	2		10	0	0	0	0	35
	15	4	10	500	500	500		15	0	0	0	0	3
	20	152	500	500	500	500		20	0	0	9	42	59
	25	4	9	500	500	500		25	0	5	7	9	20
	30	3	7	500	500	500		30	0	0	0	0	0
	37	0	0	2	4	4		37	0	0	0	0	0
3%	5	0	0	0	1	1	7%	5	0	0	0	0	0
	10	0	0	2	3	3		10	0	0	0	0	0
	15	0	2	5	5	5		15	0	0	0	0	66
	20	1	4	500	500	500		20	0	0	7	40	49
	25	1	5	157	500	500		25	0	0	0	1	0
	30	0	4	4	4	4		30	0	0	0	0	0
	37	0	0	0	1	1		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	0	0	0		10	0	0	0	0	0
	15	0	1	1	1	1		15	0	0	0	0	17
	20	1	1	1	4	4		20	0	0	0	1	1
	25	0	0	2	3	3		25	0	0	0	0	0
	30	0	0	4	4	4		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table IX: Colony count from soil site 901.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	500	500	500	500	500	5%	5	0	0	0	0	0
	10	500	500	500	500	500		10	0	0	0	0	0
	15	500	500	500	500	500		15	0	0	0	50	50
	20	500	500	500	500	500		20	0	46	50	50	50
	25	500	500	500	500	500		25	0	0	49	49	49
	30	500	500	500	500	500		30	0	2	2	2	2
	37	500	500	500	500	500		37	0	0	0	0	0
2%	5	0	50	50	500	500	6%	5	0	0	0	0	0
	10	0	200	200	500	500		10	0	0	0	4	4
	15	0	150	200	500	500		15	0	21	22	23	23
	20	3	250	250	250	250		20	0	39	40	39	39
	25	27	500	500	500	500		25	0	21	40	40	40
	30	16	51	54	500	500		30	0	27	14	14	14
	37	0	0	0	500	500		37	0	0	0	0	0
3%	5	0	0	0	33	33	7%	5	0	0	0	0	0
	10	0	0	0	50	50		10	0	0	0	0	0
	15	0	29	29	42	42		15	0	0	0	0	0
	20	1	26	42	42	42		20	0	0	0	0	0
	25	14	23	33	32	32		25	0	0	0	4	4
	30	16	17	17	17	17		30	0	0	0	0	0
	37	0	0	0	1	1		37	0	0	0	0	0
4%	5	0	0	0	28	28	8%	5	0	0	0	0	0
	10	0	0	0	41	41		10	0	0	0	0	0
	15	9	75	75	63	63		15	0	0	1	4	4
	20	28	58	64	63	63		20	0	0	9	9	9
	25	58	77	77	77	77		25	0	0	3	3	3
	30	79	11	101	104	104		30	0	0	0	0	0
	37	8	9	1	10	10		37	0	0	0	0	0

Appendix II

Table X: Colony count from soil site 902.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	500	500	500	500	500	5%	5	0	0	0	0	0
	10	500	500	500	500	500		10	0	0	0	0	0
	15	500	500	500	500	500		15	0	2	7	38	38
	20	500	500	500	500	500		20	0	11	14	14	14
	25	500	500	500	500	500		25	0	7	4	7	7
	30	500	500	500	500	500		30	0	2	7	7	7
	37	500	500	500	500	500		37	0	0	0	0	0
2%	5	0	0	0	13	13	6%	5	0	0	0	1	1
	10	0	10	10	38	38		10	0	0	0	6	6
	15	3	45	500	500	500		15	0	18	21	21	21
	20	13	115	125	120	120		20	0	7	9	11	11
	25	24	40	70	500	500		25	0	9	11	11	11
	30	27	50	50	50	50		30	0	4	9	9	9
	37	0	1	1	1	1		37	0	0	0	0	0
3%	5	0	0	0	0	0	7%	5	0	0	0	0	0
	10	0	0	0	13	13		10	0	0	0	0	0
	15	0	12	82	87	87		15	0	0	7	7	7
	20	0	57	87	87	87		20	0	1	2	5	5
	25	2	10	58	58	58		25	0	0	3	3	3
	30	7	12	13	25	25		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0
4%	5	0	0	0	0	0	8%	5	0	0	0	0	0
	10	0	0	0	15	15		10	0	0	0	1	1
	15	0	22	62	75	75		15	0	2	2	4	4
	20	0	22	52	62	62		20	0	0	0	0	0
	25	4	17	17	19	19		25	0	0	1	1	1
	30	0	9	9	9	9		30	0	0	0	0	0
	37	0	0	0	0	0		37	0	0	0	0	0

Appendix II

Table XI: Colony count from soil site 903.

Phenol treatment	Temp	NO. of colonies on the observation days					Phenol treatment	Temp	NO. of colonies on the observation days				
		3	7	14	21	28			3	7	14	21	28
0%	5	80	500	500	500	500	5%	5	0	0	0	4	4
	10	500	500	500	500	500		10	0	0	0	14	14
	15	500	500	500	500	500		15	0	3	4	6	6
	20	500	500	500	500	500		20	0	10	10	10	10
	25	500	500	500	500	500		25	0	9	11	9	9
	30	500	500	500	500	500		30	2	4	10	16	16
	37	500	500	500	500	500		37	0	3	5	5	5
2%	5	0	0	0	500	500	6%	5	0	0	0	0	0
	10	0	8	8	500	500		10	0	0	0	0	0
	15	0	500	500	500	500		15	0	0	0	0	0
	20	5	500	500	500	500		20	0	0	0	1	1
	25	3	500	500	500	500		25	0	0	3	3	3
	30	2	500	500	500	500		30	0	1	1	2	2
	37	2	5	500	500	500		37	1	1	3	3	3
3%	5	0	0	0	21	21	7%	5	0	0	0	0	0
	10	0	0	0	33	33		10	0	0	0	0	0
	15	0	25	26	34	34		15	0	2	3	4	4
	20	0	18	19	21	21		20	0	5	6	7	7
	25	1	8	9	11	11		25	0	2	2	4	4
	30	1	2	4	5	5		30	0	5	13	13	13
	37	1	3	3	4	4		37	0	2	3	3	3
4%	5	0	0	0	26	26	8%	5	0	0	0	0	0
	10	0	0	0	22	22		10	0	0	0	0	0
	15	0	8	8	17	17		15	0	0	1	7	7
	20	0	14	17	20	20		20	0	8	8	8	8
	25	3	16	17	17	17		25	0	1	6	6	6
	30	3	15	8	8	8		30	0	4	9	9	9
	37	1	3	3	4	4		37	1	2	2	2	2

Appendix III

The effect of temperature and cultivation period on growth of *Pseudeurotium zonatum*, *Chaetomium globosum* and *Chaetomium trigonosporum*

Appendix III

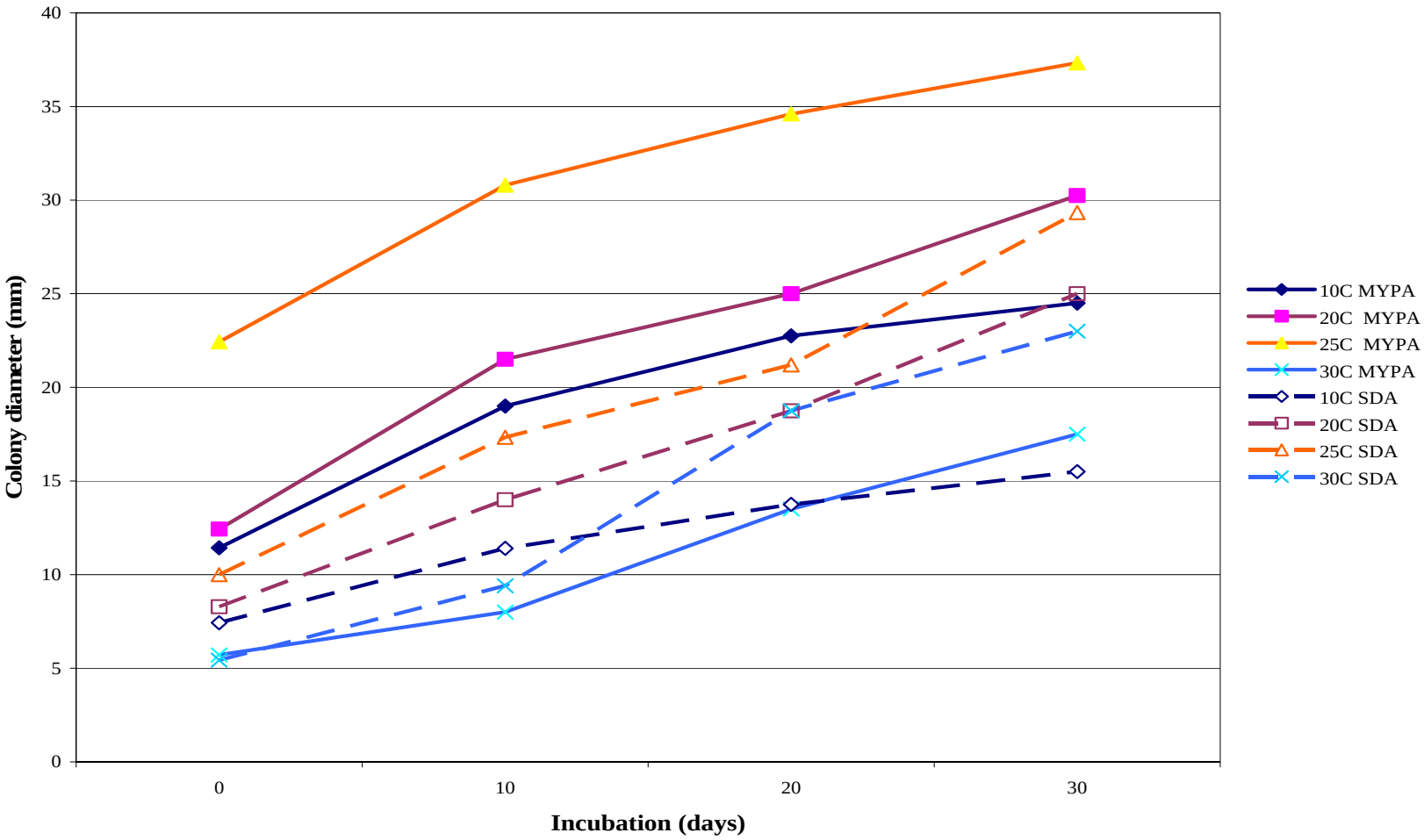

Figure I: Effect of temperature and cultivation period on growth of *P. zonatum* on MYPA and SDA

Appendix III

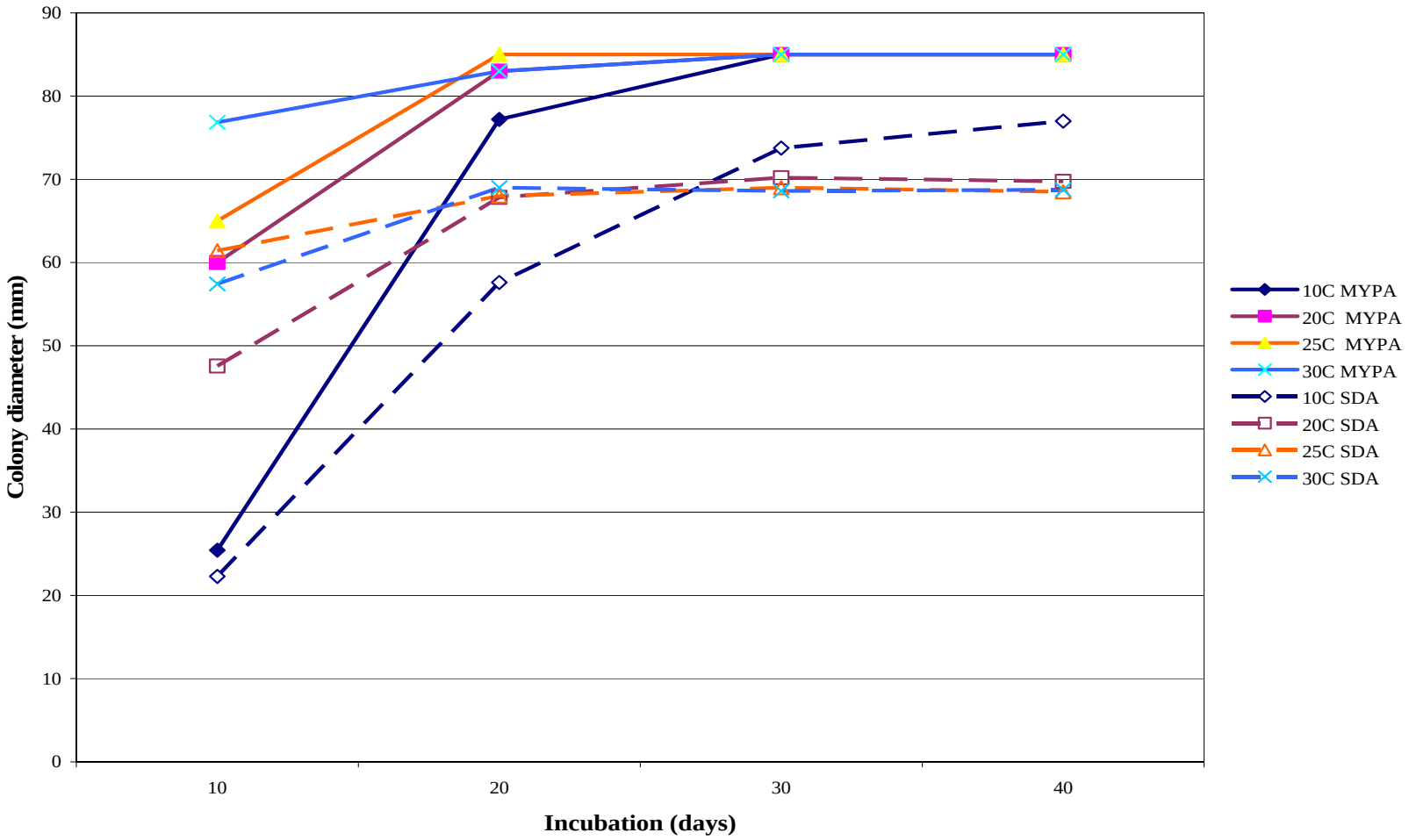

Figure II: Effect of temperature and cultivation period on growth of *C. globosum* on MYPA and SDA

Appendix III

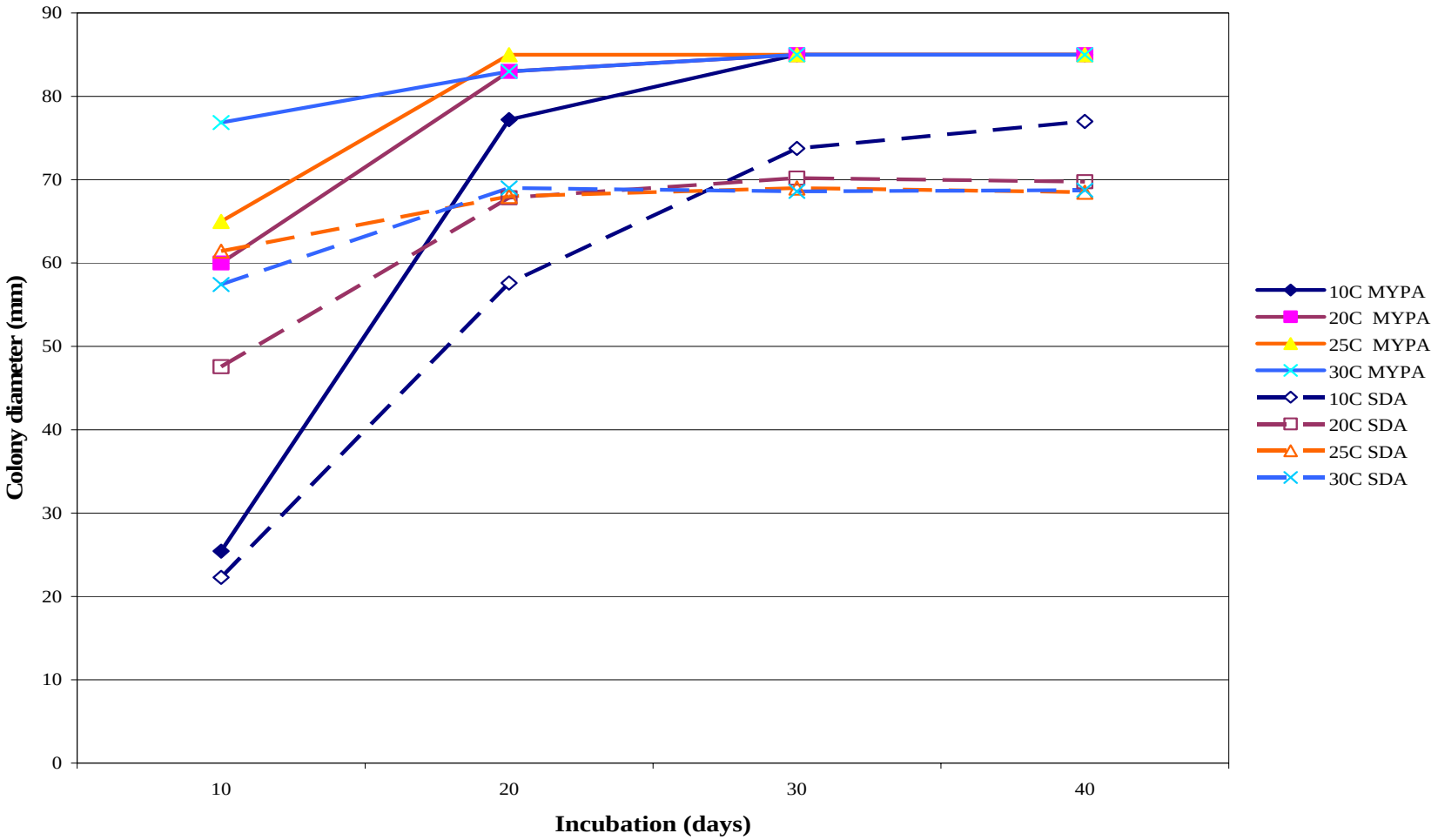

Figure III: Effect of temperature and cultivation period on growth of *C. trigonosporum* on MYPA and SDA.